ผลของแกมมา-เดลตา ทีเซลล์ในการฆ่าเซลล์มะเร็งปากมดลูกที่ได้รับยาปามิโดรเนท : การพัฒนาโมเดลเพื่อการรักษาทางภูมิคุ้มกันบำบัด

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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CYTOTOXIC EFFECT OF GAMMA-DELTA T CELLS (γ/δ) IN PAMIDRONATE TREATED CERVICAL CANCER CELLS: DEVELOPMENT OF A MODEL FOR IMMUNOTHERAPY

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มะเร็งปากมดลูกเป็นมะเร็งที่พบได้เป็นอันดับสองในสตรีทั่วโลกเป็นปัญหาสำคัญทางสาธารณสุข โดยเฉพาะผู้ป่วยที่กลับเป็นซ้ำและผู้ป่วยที่มะเร็งมีการแพร่กระจายไปยังอวัยวะอื่นๆ สาเหตุสำคัญเกิดจากการติดเชื้อ HPV การศึกษาต่างๆ เพื่อที่จะทำความเข้าใจถึงกลไกในการก่อมะเร็งของเชื้อ HPV และการพัฒนาการรักษาวิธีใหม่ๆ นั้นมีความจำเป็นต้องทำการศึกษาในโมเดลของสัตว์ทดลอง ซึ่งในการศึกษาครั้งนี้ มีวัตถุประสงค์เพื่อทำการพัฒนาโมเดลหนูทดลอง เพื่อนำมาใช้ในการทดสอบการรักษาทางภูมิคุ้มกันบำบัดด้วย แกมมา-เดลตา ทีเซลล์ การพัฒนาโมเดลหนูทดลองพบว่าการปลูกถ่ายด้วย HeLa เซลล์ ที่จำนวนน้อยที่สุดคือ 2.5×10^5 เซลล์สามารถทำให้หนูทดลองเกิดก้อนเนื้องอกได้ พบว่าค่าเฉลี่ยของขนาดก้อนเนื้องอกที่เกิดขึ้นในแต่ละกลุ่มการทดลองมีค่าเพิ่มขึ้นสัมพันธ์กับปริมาณเซลล์ที่ได้รับการปลูกถ่ายอย่างมีนัยสำคัญทางสถิติ ($R^2 = 0.98$, $y = 0.1171x+4.35$) ก้อนเนื้องอกในหนูจำนวนหนึ่งได้นำมาทำการตรวจเพื่อยืนยันว่าเป็นก้อนเนื้องอกที่เกิดจาก HeLa เซลล์จริง โดยวิธี PCR, *in situ* hybridization และ การจำแนกสายพันธุ์ของ HPV การทดสอบความสามารถของแกมมา-เดลตา ทีเซลล์ในหลอดทดลองพบว่าเซลล์มีความสามารถในการฆ่าเซลล์มะเร็งปากมดลูกได้ ความสามารถในการฆ่าจะเพิ่มขึ้นในเซลล์ที่ได้รับยาปามิโดรเนท โดยเซลล์ที่ไวต่อแกมมา-เดลตา ทีเซลล์ มากที่สุดคือ HeLa รองลงมาคือ SiHa, CaSki และ NTY ตามลำดับ นอกจากนี้การทดสอบกับเซลล์ของตัวเอง ที่ถูกกระตุ้นด้วย PHA พบว่า แม้แกมมา-เดลตา ทีเซลล์สามารถฆ่าเซลล์ดังกล่าวได้ แต่ในระดับที่ต่ำมาก กลไกในการฆ่าของแกมมา-เดลต้า ทีเซลล์ พบว่าเกี่ยวข้องกับ การกระตุ้นผ่านทาง granzyme และ perforin นอกจากนี้ยังได้ทำการทดสอบความสามารถในการฆ่าเซลล์มะเร็งของแกมมา-เดลตา ทีเซลล์ในโมเดลหนูทดลองที่ได้ทำการพัฒนาไว้แล้ว ผลการทดลองแสดงให้เห็นว่า แกมมา-เดลตา ทีเซลล์สามารถแทรกตัวอยู่ภายในก้อนเนื้องอก และชักนำให้เกิดการฆ่าตัวตายของเซลล์ได้

การศึกษานี้เป็นรายงานแรกที่แสดงให้เห็นว่าแกมมา-เดลตา ทีเซลล์สามารถฆ่าเซลล์มะเร็งปากมดลูกได้ทั้งในหลอดทดลอง และในสัตว์ทดลอง ทำให้มีความเป็นไปได้ที่จะนำแกมมา-เดลตา ทีเซลล์ มาใช้ในการรักษาทางภูมิคุ้มกันบำบัดสำหรับโรคมะเร็งปากมดลูกต่อไปในอนาคต

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MONTHON LERTWORAPREECHA : CYTOTOXIC EFFECT OF GAMMA-DELTA T CELLS (γ/δ) IN PAMIDRONATE TREATED CERVICAL CANCER CELLS : DEVELOPMENT OF A MODEL FOR IMMUNOTHERAPY
THESIS ADVISOR : ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., THESIS CO-ADVISOR : ASSOC.PROF.SUTHILUK PATUMRAJ, Ph.D., ASST. PROF. POKRATH HANSASUTA, M.D., D. Phil. (Oxon), 141 pp.

Cervical cancer is the second most common cancer found in women worldwide. It is a seriously public health problem, especially in recurrence and metastasis patients. The etiology of cervical cancer is caused by HPV infection. To understand the pathogenesis of HPV infection and cervical cancer and develop new approaches for cervical cancer treatment, the suitable of animal model is needed. Therefore, this study aims to develop a new mouse model for γ/δ T cells immunotherapy testing. The results indicated that the minimal amount of HeLa cells that could induce tumor in mouse was 2.5×10^5. The average of tumor size in each implanted mouse were significantly correlated with the increasing number of implanted HeLa cells (R^2 = 0.98, y = 0.1171x+4.35). HPV DNA in tumor section was confirmed by using PCR, *in situ* hybridization with specific HPV DNA probes, and typing with HPV DNA typing kit. The result indicated the tumor was originated from HeLa cells. The killing efficacy of γ/δ T cells was indicated that these cells have abilities to kill all tested cervical cancer cells *in vitro*. The killing efficacy increased in pamidronate treated cervical cancer cells. The most sensitive cell was HeLa cell followed by SiHa, CaSki and NTY respectively. Moreover, this result indicated γ/δ T cells had less effect to autologous PHA blast PBMC. The killing mechanism of γ/δ T cells involved with degranulation of perforin and granzyme pathway. Further investigation the role of γ/δ T cells in developed mouse model demonstrated that these cells could deposit within tumor area and were able to induce apoptosis in tumor cells.

This study was the first evidence indicating that γ/δ T cells could kill cervical cancer both *in vitro* and *in vivo*. Therefore, the development of cervical cancer immunotherapy in the future by using of γ/δ T cells could be possible.

Field of Study : Medical Microbiology Student's signature…….....

Academic year : 2009 Advisor's signature…….…….....

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LIST OF ABBREVIATIONS

AIDS	=	Acquired immune deficiently syndrome
APC	=	Antigen presenting cell
ASC	=	Atypical squamous cells
CAMs	=	Cell adhesion molecules
CIN	=	Cervical intraepithelium neoplasia
CMI	=	Cell mediated immunity
CMIR	=	Cell mediated immune response
CMFDA	=	5-chloromethylfluorescein diacetate
COPV	=	Cotton tail rabbit papillomavirus
CTL	=	Cytotoxic T lymphocyte
DAB	=	3,3′ diaminobenzidine
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
dsDNA	=	Double strand Deoxyribonucleic acid
ELISA	=	Enzyme linked immunoassay
FBS	=	Fetal bovine serum
FITC	=	Fluorescein isothocyanate
FPP-synthase	=	Farnesylpyrophosphate synthase
H	=	Hours
H&E	=	Haematoxylin and eosin staining
HPV	=	Human papillomavirus
HR	=	Horseradish peroxidase
HSIL	=	High-grade squamous intraepithelial lesion
IFN-γ	=	Interferon-γ
IP	=	Intraperitoneal
IPP	=	Isopentenyl pyrophosphate
IL	=	Interleukin
IRF-3	=	IFN-regulatory factors-3
ISGF3	=	Interferon stimulated gene factor 3
ISRE	=	Interferon-stimulated response element

LIST OF ABBREVIATIONS (Continue)

LC	=	Langerhans cell
LDH	=	Lactate dehydrogenase
LCR	=	Long control region
LMP	=	Low molecular mass protein
LSIL	=	Low-grade squamous intraepithelial lesion
mAb	=	Monoclonal antibody
MACS	=	Magnetic activating cell sorter
MCP-1	=	Monocyte chemoattractant protein-1
MEM	=	Minimal essential medium
MEP	=	Mevalonate pathways
MHC I	=	Major histocompatibility complex class I
MHC II	=	Major histocompatibility complex class II
ml	=	Milliliter
mRNA	=	Messenger RNA
nBP	=	Nitrogen bisphosphonate
NF-κB	=	Nuclear factor kappa B
NK	=	Natural killer cell
OD	=	Optical density
ORF	=	Open reading frame
PBMC	=	Peripheral blood mononuclear cell
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
pRB	=	Retinoblastoma protein
RPMI	=	Rosewell Park Memorial Institute
TAP	=	Transporter of antigens peptides
TNF-α	=	Tumor necrosis factor-α
TCR	=	T cell receptor
Th-1	=	Helper T lymphocyte-1
TRAIL	=	Tumour necrosis factor related apoptosis ligand
Th-2	=	Helper T lymphocyte-2

LIST OF ABBREVIATIONS (Continue)

TGF-β	=	Tumor growth factor–β
TUNEL	=	Terminal deoxynucleotidyl transferase dUTP nick end labeling
μM	=	micro Molar
μl	=	micro litter
VLP	=	Viral like particle

CHAPTER I

INTRODUCTION

Cervical cancer is the second common cancer among women worldwide. It causes a big public health problem continuously in many countries. More than 80% of all cases occur in women living in developing countries including Thailand [1, 2]. Epidemiology and molecular biology studies indicated that the high risk human papillomaviruses (HPVs) are recognized as the major etiology of this cancer. Up to date, over 100 types of HPV are recognized which 30 - 40 types are associated with anogenital infection. Approximately 30 types of anogenital HPV are in high risk HPV group especially HPV-16 and 18, which are the most prevalence types found in cervical cancer worldwide [3, 4].

Anogenital HPV infections are usually common and the cumulative lifetime incidence is estimated to be 80-85 % [5]. The fact that most infections are cleared and that low-grade cervical intraepithelial neoplasia (CIN) lesions often regress spontaneously, indicates that in the majority of individuals the immune system succeeds in eliminating the virus before malignant disease can develop. In natural HPV infected patients, some patients have a serum antibody against L1 capsid protein [6, 7]. Although, the serum antibody titers at peak are quite low, a report in animal model reveled that this antibody can protect the animal from challenging with large doses of infectious viruses [8].

Unfortunately, when HPV infection establishes these serum antibodies are unable to eliminate the infected cells. The clearance of HPV-infection likely requires T

cells specific for the viral antigens [9-14]. Nevertheless, most studies demonstrated that cell mediated immunity (CMI) plays an important role in controlling HPV-infection and cervical cancer development [15-18]. It is noteworthy that the time from infection to appearance of lesions or elimination of infection is highly variable from weeks to months, especially high risk HPV (HPV-16 and 18) can persist for long time[19]. This evidence implies that HPV can escape from host immune system. There are many explanations why immune system fails to eliminate HPV infection. Since HPV infections are local and non-lytic infection. Thus antigen presentation by HPV-infected epithelium cell must be low. Due to low antigenic presentation, the migration of antigen presenting cells (APCs) to the sites of infection is inhibited. Hence no T cells are activated. This evidence supporting by clinical observation, which cervical lesions often regress after biopsies have been shown [20], indicated that tissue disruption, due to the biopsy, attracts APC to the infected sites. Although, in the absent of cells cytolysis by HPV infection, responding of CMI to HPV infection still depends on major histocompatibility complex class-I (MHC-I) restriction. Unfortunately infection with this HPV is able to down regulate the MHC-I expression resulting in an impairment of MHC restricted, cytotoxic T cells mediated tumor specific immunity [21, 22]. In addition, HPV have a mechanism to inhibit the expression of interferon-α (IFN-α), the type-I interferon that plays roles in host antiviral defense system[23].

Although current standard therapeutic strategies for cervical cancer including surgery, radiotherapy or chemotherapy are usually effective for achieving initial disease control. However management of patient with recurrence and metastatic disease is still unsolved. After surgery or radiotherapy treatment approximately 10-20% patients may develop recurrence and/or distant metastases, and usually

associate with poor prognosis [24]. Hence new forms of therapy for recurrent or metastatic cervical cancer are needed. The view that the immune system itself might be exploited for the treatment of cervical cancer is not new. Interestingly to date the overwhelming majority of reports in this regard have focused on the adaptive immune response to cervical cancer with specific HPV antigens. Several studies attempted to induce tumor specific CD8+ α/β T cells (CTLs) using HPV specific antigens while some studies developed tumor specific immune responses using dendritic cell based vaccination strategies [25-27]. As mentioned above, HPV positive cervical cancer cells can down regulate expression of MHC molecules. Thus, tumor cells expressing few or no MHC molecules might selectively escape recognition of CD8+ CTL. These latter findings emphasize the importance of developing immunotherapy strategies that do not depend on classical MHC restricted antigen processing and presentation.

Recently, several studies reported the ability of the gamma-delta (γ/δ) T cells in the role of tumor immunotherapy because these cells can act as immunological effector cells against several tumor cells [28-33]. γ/δ T cells represent a small subset of T cells (2-10% of T cell population) that possess a distinct T cell receptor (TCR) on their surface. Majority of T cells has a TCR composed of two glycoprotein chains called α- and β- TCR chains. In contrast to γ/δ T cells, the TCR is made of one γ-chain and one δ-chain. This group of T cells is usually much less common than α/β T cells. They are found localized in the mucosal associated lymphoid tissue, tongue, esophagus, trachea, lungs, genital epithelia and skin [34].

Unlikely to α/β T cells, γ/δ T cells recognize non-peptide antigens and exhibit a non MHC restriction. Recently, relevant antigens that activate the γ/δ T cells

have been identified. It is non peptidic phosphorylated group, the intermediates of the non-mevalonate pathway found in bacterial isoprenoid biosynthesis and mevalonate pathway for isoprenoid biosynthesis found in eukaryotic cells [35]. Both pathways generate isopentenyl pyrophosphate (IPP), the intermediate products that are an important antigen for γ/δ T cells. Interestingly, certain malignancy cells produce high concentration of IPP, which can be recognized by the TCR of γ/δ T-cells as a tumor antigen [35, 36].

Intracellular levels of IPP can be induced by using the nitrogen bisphosphate (nBP) compounds, which are the chemical drugs use for treatment of osteoperosis and bone metastasis [37]. This compound causes accumulation of the IPP in the cells by inhibiting the function of farnesylpyrophosphate synthase (FPP-synthase), a key enzyme at the down stream of IPP product.

The role of nBP drugs to induce proliferation and enhance the cytotoxic function of γ/δ T cells to cancer cells has been shown in several experiments. For example, in the case of multiple myeloma and lymphoma, the nBP drugs (alendronate, ibandronate and pamidronate) are able to induce significant expansion of γ/δ T cells in peripheral blood mononuclear cell cultures of healthy donors. Moreover, the pamidronate activated γ/δ T cells showed the cytotoxicity against lymphoma and myeloma cell line [38]. However, a few observation has been documented that increasing number of wart infiltrating γ/δ T cells can be observed in HPV-6 infected genital wart and suggested that this cell may have an important role in controlling of HPV infection [39].

As noted above, cervical cancer is still the major public health problem. It is the one leading cause of cancer death in women worldwide. The problems in recurrence and/or distant metastases patients are the driving factor for many researchers to develop new treatment approach for this cervical cancer. However, the one obstacle for study in development of pathogenesis and cervical cancer treatment is the lack of suitable *in vivo* model. The reason that impedes in developing an animal model is that cervical cancer is caused by HPV infection. Unlike other viruses, HPV is highly host-specific, unable to propagate in normal cell culture or *in vivo* model [40]. It replication cycle requires the differentiation process of keratinocytes [41]. Until now, the interaction of virus and host is not well understood. Many attempts have been made using several means to develop animal model. One example is direct inoculation of HPV extracted from naturally human lesion to human neonatal foreskin and then grafted underneath the renal capsule of athymic mice [42]. Since it was transplanted into the renal capsule observation of the any changes such as angiogenesis in tumor area are impracticable. Another, attempt was done by creating transgenic mice model carrying HPV E6-E7 gene [43]. This experiment indicated that HPV E6 and E7 are very important to induce proliferation and accelerate progression of cells to invasive cancer. Nevertheless, transgenic mice model does not study the context of integration of human tissue viral infection, tumor that grown on transgenic mice is not human tissue. Hence, observation study the role of new agent such as new drugs, vaccine testing, or immune surveillance by using this transgenic mice model may unaccountable. Therefore, the ideally animal model for cervical cancer and HPV should breakthrough a limitation as mentioned above. The mice model should easily be able to show the beginning steps of human tumor establishment such as increased vascularization.

Altogether, it is very interesting to evaluate the role of pamidronate, one of nBP drugs, in activating γ/δ T cells to kill cervical cancer cells both *in vitro* and *in vivo*. An attempt in establishing animal model carrying cervical tumor is also included.

Hypothesis

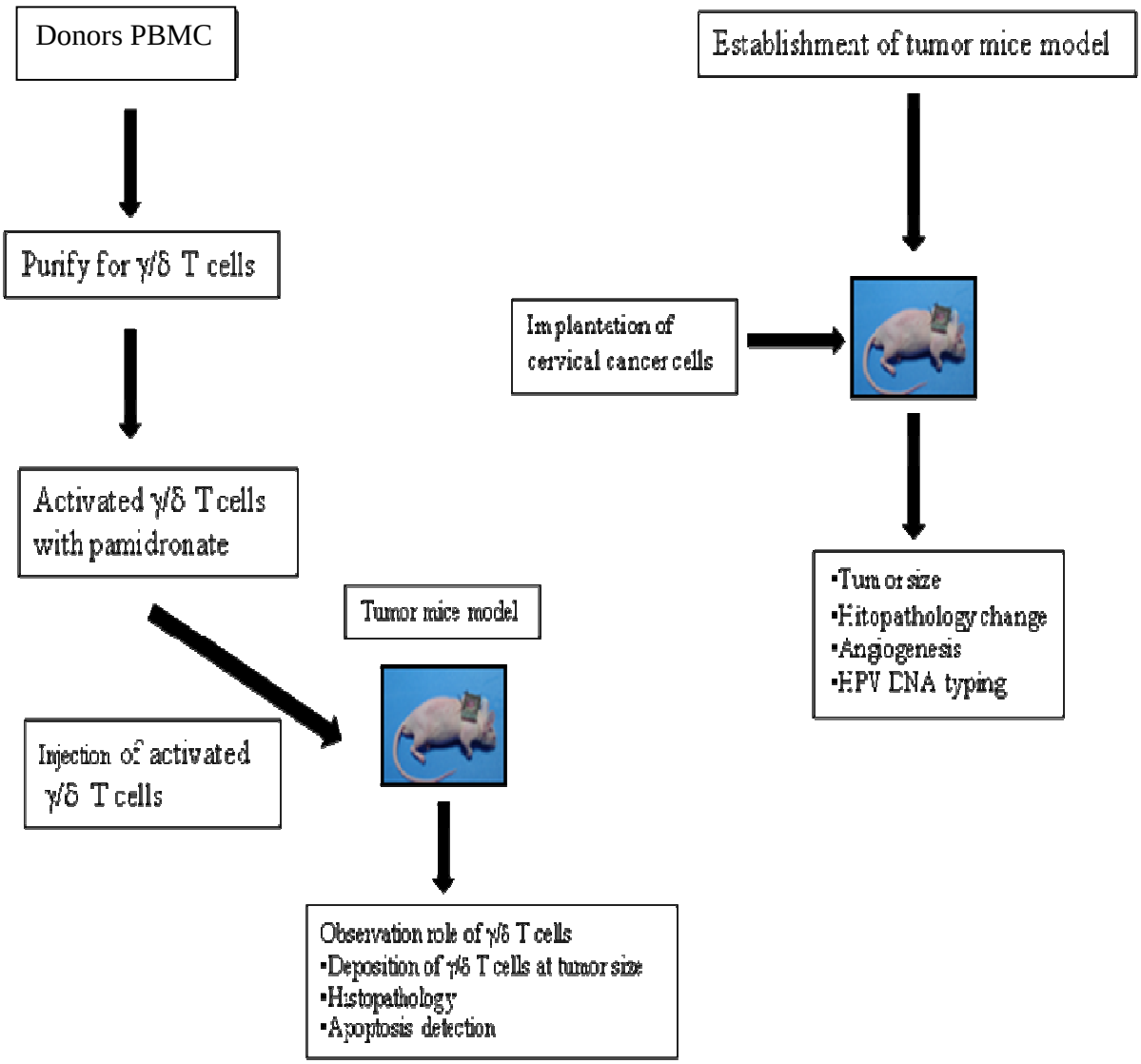
Cervical cancer cells treated with pamidronate can activate γ/δ T cells killing activity.

Objectives

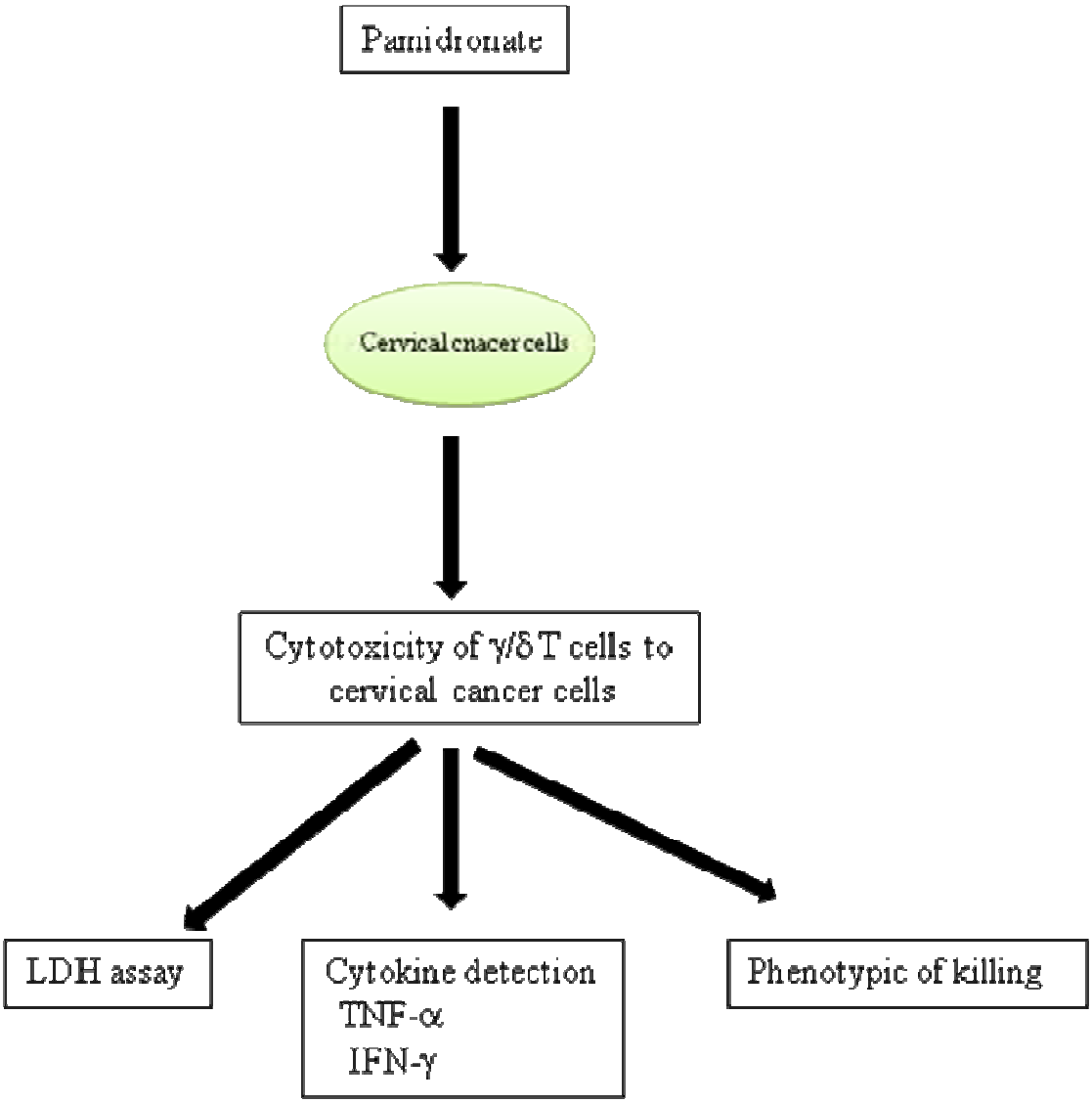
1. To establish the animal model baring cervical cancer cells
2. To investigate the cytotoxicity of gamma delta T cells to pamidronate treated cervical cancer cells both *in vitro* and *in vivo*
3. To define the gamma delta T cells cytotoxic mechanism involving in cell lysis of pamidronate treated cervical cancer cells

Conceptual frame work

In vivo



***In vitro* study**



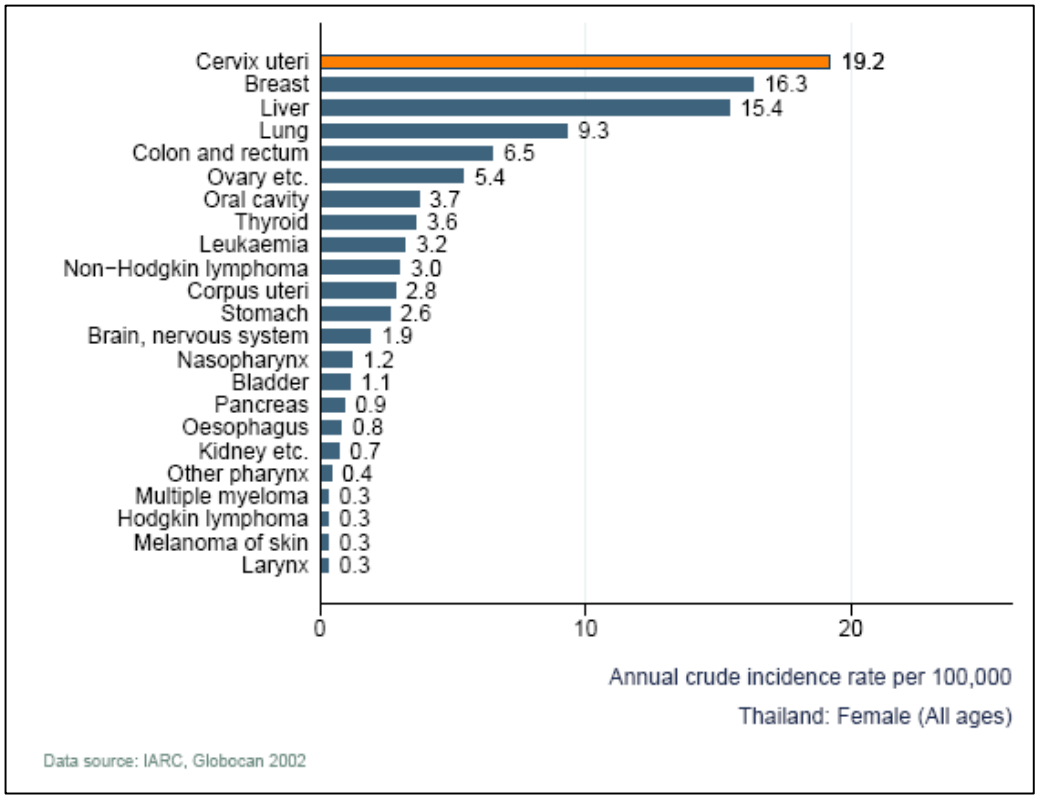
CHAPTER II

REVIEW AND RELATED LITERATURES

Cervical cancer: overview

Cervical cancer is a malignant tumor that occurs in the tissue of the uterine cervix or cervical area. It may begin as asymptomatic pre-cancerous lesions and usually develops invasive cancer gradually over many years. According to World Health Organization (WHO), cervical cancer is the second most cancer found in women worldwide, with approximately 500,000 new cases and about 240,000 deaths in each year [1, 44]. Almost 80 % of the cases and death found in developing country [44]. In Thailand, the data reported by WHO in the year 2007 indicated that cervical cancer is the first cancer found in Thai women (Figure 1) [2].

Several epidemiologic evidences show that cervical cancer behaves as a sexually transmitted disease [45] and many risk factors may involve in developed or accelerated progression of the disease such as age at firs intercourse, multiple sexual partners, smoking and contraceptive used, etc [46-49]. At present, the molecular biological studies are well documented that almost 100% of cervical cancer cases worldwide are caused by persistent infection of human papillomavirus (HPV). Several prevalence studies in various stages of cervical cancer (from precancerous lesion to invasive cervical cancer) demonstrate that the proportions of HPV-DNA range from 40-99% in low grade to high grade cervical cancer respectively [50-53], but only 15% of HPV-DNA can be detected in normal cytology women.



WHO/ICO Information Centre on HPV and Cervical Cancer (2007) [2].

Figure1. Cervical cancer incidence in Thailand compared to other cancers in women of all ages.

HPV: virology

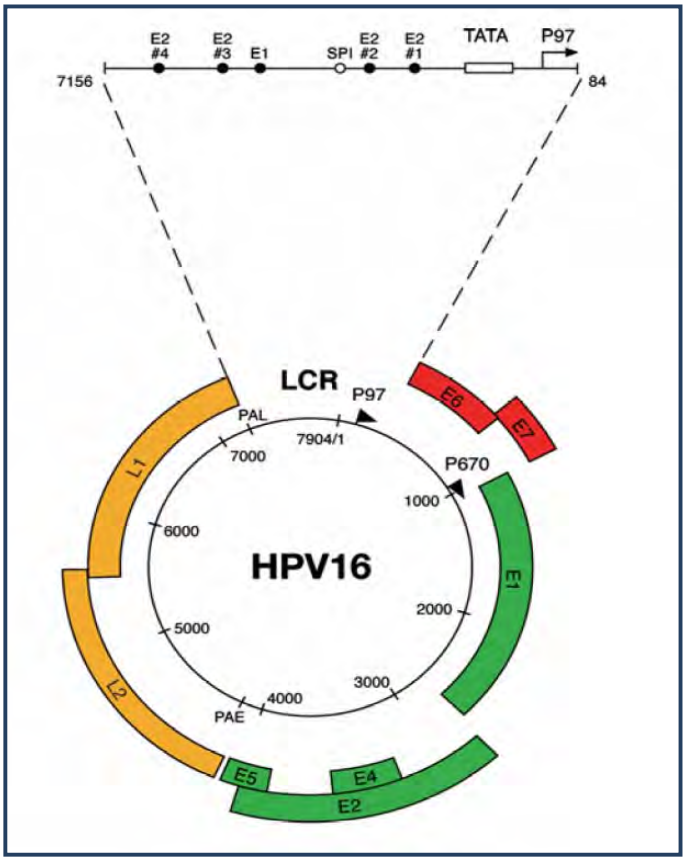
HPV, a group of oncogenic small DNA virus belongs to the *Papillomaviridae* family. Papillomavirus can induce warts (papillomas), benign tumor and malignancy in many different tissues of higher vertebrate including man [54]. However, HPV is human specific, and unable to infect another species.

The HPV genome is a circular dsDNA approximately 7.9 kb in length. The open reading frames (ORF) of HPV genome are located on one strand. The genome is separated into three functional regions (Figure 2). The first is a noncoding upstream regulatory region, this region known as long control region (LCR). The region contains

the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. The second is an early region, consisting of ORFs E1, E2, E4, E5, E6, and E7, which encode for early proteins that are involved in viral replication and viral oncoprotein. The third is a late region, which encodes for two structural proteins of the virus that are, major capsid protein (L1) and minor capsid protein (L2) [41].

HPV can be classified in to various types based on degree of genetic relatedness of L1 ORF. If nucleotides sequence of L1 ORF shows more than 10% different with previously described type, it was classified as a new type. Differences between 2-10% homology define a subtype and less than 2% is a variant [55]. Up to date, over 150 types of HPV are recognized which 30- 40 types are associated with anogenital infection. Approximately 30 types of anogenital HPV are in high risk HPV group, especially HPV-16 and 18 which are the most prevalent types found in cervical cancer worldwide [3, 4, 56-58]

Besides typing, HPV also divided on the basic of the site of infection, resulting in two main HPV groups: cutaneous and mucosal. The majority of cutaneous group (such as HPV-1,-5, 8,9,12, 15 and 21) associates with skin lesion causing, skin wart and common wart. The mucosal group is found in the oral mucosa, respiratory tract, conjunctiva, and anogenital tract. Among those cause anogenital tract infection also be divided in two groups corresponding to their pathogenicity in developing of cancer i.e., high risk group (such as HPV-16, 18, 52 and 58) and low risk group (such as HPV-6, 11, 42 and 44).



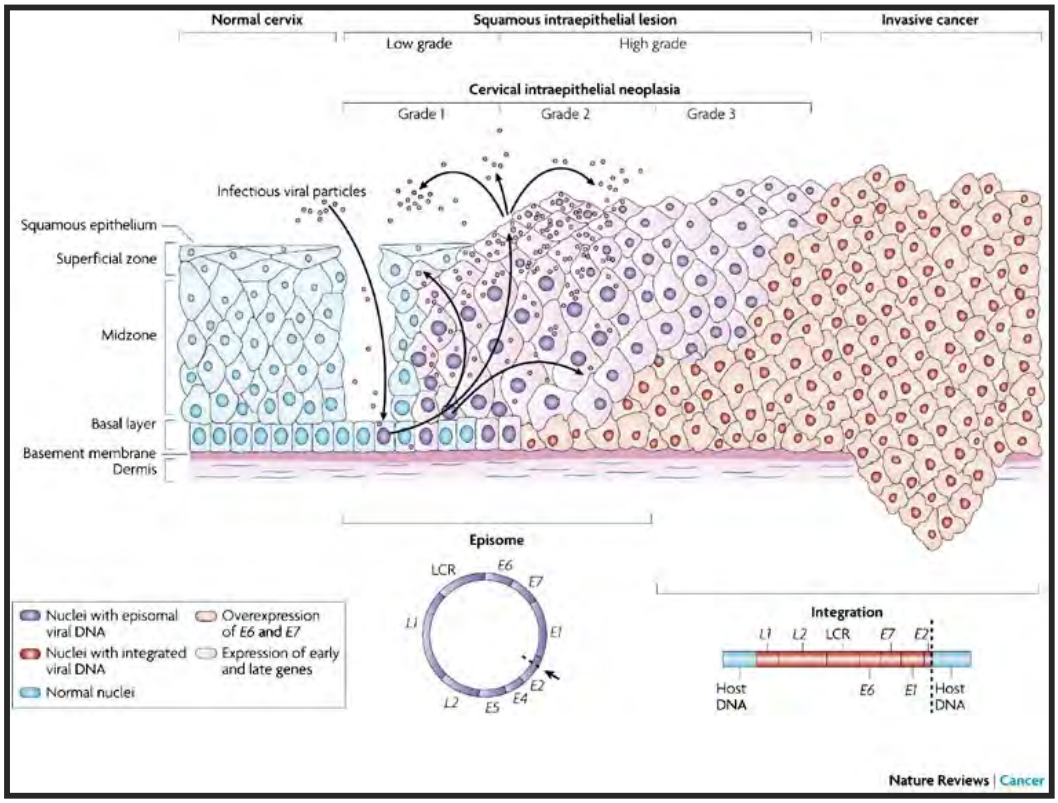
Clinical Science (2006) [59].

Figure 2. Genome organization of HPV. All HPVs are approximately 7.9 k b in size and consist of covalently closed double stranded DNA, with a highly conserved organization among all known PVs. The HPV genome contains a 400 –850 bp long noncoding region (long control region, LCR, or upstream regulatory region, URR), which contains all regulatory proteins.

HPV life cycle and replication

HPV infection of cervical epithelium cells after sexual transmission generally occurs via damage of epithelium. The basal layer of epithelium, probably stem cell is the main target of HPV infection. The virus can penetrate and infect in to the epithelium layer via micro-abrasions (Figure 3). The specific viral receptor on host cells surface is not yet known however, heparin sulphate and proteoglycan on epithelium cells may be a candidate of HPV receptors [60].

Once HPV has already infected the cells, the life cycle can be distinguished in to two stages. Firstly, the nonproductive stage which is usually found in early infection; the virus maintains its genome in the form of episomal DNA or episome [61]. The E1 and E2 are the viral proteins that play role in maintaining the vial DNA as episome [62]. HPV DNA can be distributed into two daughters via division of basal cells. Thus one cell remains at the basal layer or stem cell creating a latent infection meanwhile, another cell can move and differentiate through the upper layers of epithelium. Secondly, the productive stage is strictly associated with the terminally differentiation of epithelium cells. In these cells, the virus switches to a rolling-circle mode of DNA replication and amplifies its genome to higher copy number, expresses the L1 and L2 gene for major and minor capsid proteins, next the mature virions then are assemble and released from epithelium cells within the superficial epithelial cells [63].



Nature Reviews Cancer (2007) [63].

Figure 3. HPV infects epithelium layer through damage epithelium layer. The viral DNA replicates in the lower layers of epithelium. In the nonproductive stage, early (E1-E7) genes are expressed and viral DNA replicates from episome. The E1 and E2 are the proteins that support replication of viral genome as episome. The productive stage occurs in the upper layer (the mid zone and superficial zone). In this area, late gene (L1 and L2) are expressed and immense DNA replication (up to 10,000 copies/cell). L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection.

HPV and pathogenesis of cervical cancer

The mechanism of HPV induction of cancer becomes better understood when the viral genome organization is known. In the benign or pre-neoplastic lesion, HPV-DNA is not integrated to the host DNA but rather persists as an extrachromosomal DNA (episome). However, in malignant lesions or in different cell lines that are derived from cervical cancer, the HPV-DNA integrates into the host chromosome. Integration of HPV-DNA in host chromosome may occur in one or many copies, and no sites specificity for integration to host however, most of integration occurs in the E1/E2 region of HPV genome [64]. The E2 protein acts as a regulatory protein and plays an important role in regulating the expression of E6 and E7 protein

The E6 and E7 proteins are the oncoproteins, they have malignant transformation activities. The E6 protein is able to bind p53 tumor suppressor protein and accelerate degradation of p53 protein via ubiquitin protease system [64]. The E7 protein is able to bind retinoblastoma protein (pRB), a product of the tumor suppressor gene Rb-1. During the transition to the S phase, pRB is phosphorylated by cdk, resulting in the inactivation of its cell cycle regulatory functions. The cellular transcription factor E2F is preferentially bound to the dephosphorylated form of pRB, and in complex with pRB can not activated transcription. The complex formation of E7 and pRB results in the release of E2F, allowing it to function as a transcriptional activator to cellular genes involve in cellular DNA synthesis and progression into the S phase of the cell cycle [64]. Thus binding of E6 and E7 proteins to p53 and pRB, respectively, offers an appealing, direct biochemical mechanism to explain HPV oncogenesis (Figure 4). In addition, some study indicated that progression of cervical

cancer involved in the instability of the chromosome that induced by E6 and E7 proteins [65].

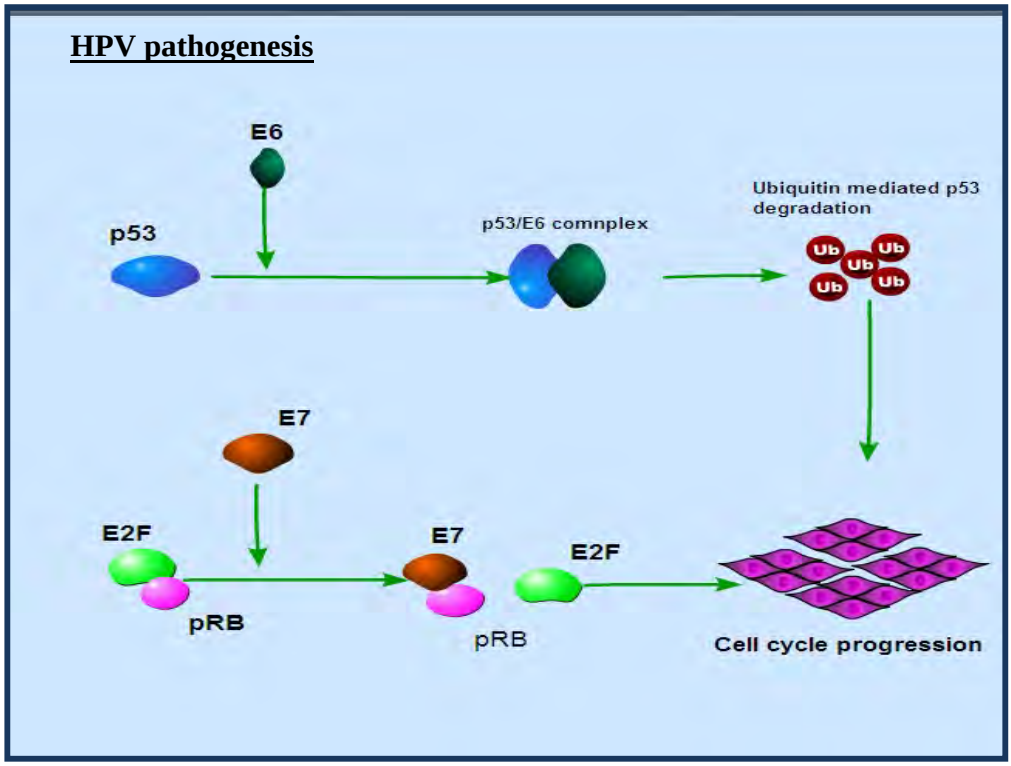


Figure 4. Pathogenesis of cervical cancer, E6 protein of HPV is able to form complex with p53 protein and accelerate dragadation of p53 via ubiquitin protease system. E7 protein can disrupt the complex between pRB and E2F. The complex of E7/pRB releases the E2F to freely and continue its function as a transcription factor to activate DNA synthesis and cell proliferation.

Grading of cervical cancer

It is well known that invasive cervical cancer originates from precancerous lesion stage. The former system that used for grading this precancerous stage was known as cervical intraepithelium neoplasia grade 1-3 (CIN 1-3). However, the new system was introduced by Bethesda system in 1988 and update again in 2001[66]. This system classified squamous cell abnormalities into four categories as following:

i. Atypical squamous cells (ASC)
   of undetermined significance (ASC-US)
   cannot exclude HSIL (ASC-H)
ii. Low-grade squamous intraepithelial lesion (LSIL)
   encompassing: human papillomavirus/mild dysplasia/cervical intraepithelial neoplasia (CIN) 1
iii. High-grade squamous intraepithelial lesion (HSIL)
   encompassing: moderate and severe dysplasia, carcinoma *in situ*; CIN 2 and CIN 3
iv. Squamous cell carcinoma

Low grade squamous intraepithelium lesions are characterized by increased proliferation of basal epithelial cells leading to the typical morphological changes. This stage, usually associated with several HPV types including high risk and low risk types. Our previously observations indicated the approximately 40 % is of LSIL harbored high risk HPV group especially HPV -16 [53]. HPV-DNA in this is stage usually found in episomal form and rarely or undetectable E6 and E7 proteins

[67]. Most of low grade lesion usually regresses within 3 years. Approximately 25% of low grade lesion can progress in to high grade lesion within 2-4 years and risk of progression associates with high risk HPV [68]. In opposite to LSIL, more than 70% of HSIL were infected with HPV-DNA [51]. With high expression of oncoproteins E6 and E7. When integration of HPV-DNA into host chromosomes occurs via disrupted of E2 gene the infection seems to be irreversible[69]. This explains why spontaneously regression from the high grade lesion is rare and usually progression to be invasive squamous cell carcinoma [70].

Host immune response and evading of HPV

Overview of the Immune System

The host defense system is the accomplice between innate immune system (such as skin and epithelium barriers, phagocytic mononuclear cells and soluble protein) and adaptive immune system (such as B-lymphocyte, helper T-lymphocyte, and cytotoxic T-lymphocyte). Innate immune system is the first line of defense mechanism. It will detect the invade microorganism and also abnormal cell such as tumor cell. The innate immune cells are able to distinguish the microbes from self antigens by recognition of specific structures found commonly in microbes that know as pathogen-associated molecular patterns. No specific memory cell after antigen recognition is established but this innate cells act as accessory cells to activate adaptive immune cells. In contrast to innate immune cells, adaptive immunity has specificity to distinct foreign antigens and recognition is able to generate specific memory cells that can response more energetically to the known antigens. These adaptive immune

responses develop later by activation of the lymphocytes. There are two types of adaptive immune response, humoral immune response (HMIR), and cell mediated immune response (CMI).

In HMIR, this mediated by antibodies that are secreted by activated B-lymphocytes. The antibodies have ability to neutralize specific antigens that result in abolishing the infectivity of microbes and eliminating the microbes by various mechanisms. B-lymphocytes can be directly activated by recognizing the native form of foreign antigens and differentiate to be plasma cells, antibody secreting cells. Besides direct activation, B cells can be activated by cytokines releasing from activated T cells. Activated T cells are primed to antigens presented by antigen presenting cell (APC).

CMIR is mediated by T-lymphocytes; T-cells recognize antigens that process and present with the MHC molecule on cell surface of APC. There are two major subsets of T cells, distinguished by different surface markers, CD4+ and CD8+ T cells. CD4+ T cells recognize exogenous antigens that process and present with MHC-II on APC cell surface; CD8+ T cells recognize endogenous antigens that process and present by MHC-I on APC cell surface. An interaction between T cells and APC is very sophisticate because only antigen alone can not activate T or B cells. The outcome of activation resulted from several accessory molecules on both lymphocytes and APC.

Immune response to HPV

HPV infection occurs when virus enters into the basal cell through the disruption of epithelium cell and the replication are absolutely restricted to differentiating program of epithelium cell [6]. Incubation period from infection to lesion manifestation can vary from weeks to months and interval between acquisitions of HPV infection through malignant progression usually takes at least 10 years or longer [19, 71, 72]. These imply that HPV can escape from the host immune system. However, most of the cervical HPV infection in the cases of low-grade lesion often regress spontaneously, indicates that in the majority of individuals immune system is able to eliminate the virus before cancer develops [73]. This evidence supported by many studies indicated that HPV infected patients, but not all, have a serum antibody against to L1 capsid protein [6, 74, 75]. In addition, the role of antibody to protect papillomavirus infection has been demonstrated in rabbit model by directly intravenous injection of cotton tail rabbit papillomavirus (COPV). This model revealed that the antibodies to COPV can be detected after immunized with this virus. Moreover, the immunization rabbits were completely tolerance to subsequent viral challenge by abrasion of the epithelium [76].

However, when HPV infection establishes these serum antibodies are unable to eliminate the infected cells. The clearance of HPV-infection seems to be requires T cells specific for the viral antigens. The evidences for the role of the CMIR in HPV infection and cervical cancer are demonstrated. The prevalence of persistent HPV infection and HPV positive lesions greatly increased in immunosuppressed individuals such as epidemodysplasia verruciformis, organ transplant recipients and

AIDS patients [6, 9, 11, 14, 77]. Although, the major role of CMIR in controlling and clearing the HPV infection in precancerous lesion are irrefutable, the characteristics of an effective cell mediated immune response are poorly understood.

Immune evading by HPV

HPV is one example virus that successes in induction of chronic infection. Infections have no systematic sequelae and rarely kill the host cell but, periodically shed large amounts of infectious virus for transmission to naïve individuals.HPV uses many different strategies to evade immune recognition as the following.

Squamous epithelium cells act to protect HPV from the immune cells

As noted that, HPV replication cycle is absolutely associated with differentiation of keratinocytes. In the basal layer epithelium, the HPV genome usually remains in the episomal form and the replication cycle appears to depend on cell cycle. Thus only few viral copies (50-100 copies/cell) usually be found within infected cell. In this situation, expression of viral oncoproteins, E6 and E7 are rarely or undetectable [78]. Thus, the infected basal cells act as a progeny (or transit amplifying cells) that supports for minimal episomal DNA replication. When keratinocytes enter into the differentiation stage, the massive up regulation of viral oncogene (E6 and E7) and late gene (L1 and L1) and huge DNA replication at least 1,000 copies/cell can be detected within differentiated keratinocytes [79]. The viral assembly appears only on the upper layers of differentiated squamous epithelium cell. Therefore, very rare or none of intact viral or viral antigens can be APCs such as macrophage, dendritic cell (DC), or

langerhans cells (LC). There is no cytolysis by HPV infection hence, no chance or probably rare for the immune cells outside the skin tissue can be recognized the viral antigens.

HPV aborts dendritic cells and langerhans cells

According to immunological theory, when HPV infection occurs, the professional APC should recognize HPV infected epithelium cells, since normally cervical tissue have resident DC and LC [78]. Entry of most viruses into epithelium cell usually generates signal to activate DC and LC by inducing inflammation environment. The DC and LC can up-regulate MHC class I and II molecules as well as co-stimulatory molecules such as B7, CD80, CD86 and chemokine receptor-7 (CCR-7), and increase secretion of cytokine such as IL-12. All of these are the molecules help for stimulating the naïve $CD4^+$ and $CD8^+$ T cells in the lymph nodes [78]. However, in the case of HPV infection, LC can not generate and present HPV specific epitopes when it was incubated with viral like particle (VLP) of HPV 16 L1 protein [80]. In contrast, DC can be activated and induce specific T-cells to VLP-L1 [81]. Since HPV usually remains with in the epithelium cell and assembly occur in a cell that will terminally differentiate. Thus the dying of epithelium cells will then be the normal process of cell remodeling, which is not caused by cell damaged or cell stress. Hence, uptake of the un-inflammation epithelium cells by DC and LC lead to release immunosuppressive cytokines such as tumor growth factor –β (TGF-β) IL-10 and IL-13. These results in adaptive immune ship toward a Th-2 response rather than Th-1 response [82].

Down regulate interferon gene expression

Type I interferon (IFN), interferon-α and interferon-β (IFN-α, IFN-β) are molecules that virus infected cells secrete for combating to viruses by directly degrading the viral and cellular mRNA resulting in inhibition of the viral replication. Moreover, IFN acts as immunostimulatory molecule that stimulate expression of MHC-I on infected cells, facilitation the presentation of viral epitopes and increasing the cytotoxic capacity of the T-lymphocytes [83]. However, most of DNA viruses have and escape mechanism that can overcome the role of type I INF, which HPV is include. Some reports indicated high risk HPV is able to down regulate expression of IFN-α inducible gene [78]. While others showed that HPV 16 E7 oncoprotein can inhibit the induction of IFN-α inducible genes. Expression of E7 correlates with the loss of formation of the interferon stimulated gene factor 3 (ISGF3). ISGF3 is a *trans*-acting, trimeric complex composed of STAT1, STAT2, and the p48 DNA-binding protein. ISGF3 translocates to the nucleus where it binds to a *cis* element called the interferon-stimulated response element (ISRE), resulting in initiation of transcription [84]. Moreover, in other DNA micro array study of gene expression also showed that HPV16 E6 and E7 are able to abort expression of IFN response genes such as NF-κB stimulating genes and cell cycle regulation genes [85, 86]. The E6 protein of HPV also appears to disrupt the IFN pathway by biding to the IFN-regulatory factors-3 (IRF-3), a family of transcription factors that mediate both virus and IFN signaling pathways, which are involved in anti-viral defense, immune response and cell growth regulation. They play an essential role in regulating expression of type I IFN. The complex of E6 and IRF-3 result in inhibit it trans-activation function, hence no transcription of IFN-β mRNA was found [87].

Modulation of antigen presentation

It is well known that the immune system combats invading microorganism by neutralizing antibody or directly killing the infected cells with CMI response. Antigen recognition occurs when T cell can recognize the antigens that process and present by MHC class I and II on the surface membrane of APC. However, HPV has ability to escape this by down regulation of MHC molecules and inhibiting expression and processing through MHC molecules. There is an experiment demonstrated that E7 oncoprotein of HPV 18 involved in suppression of the MHC heavy gene promoter and also the transporter of antigens peptides (TAP) and proteosome subunits low molecular mass protein (LMP), protein that involved in antigen processing and presentation [88]. Thus the poor antigen presentation due to down-regulation of antigen presentation machinery results in the induction of HPV-specific T cell anergy.

Inhibition of cytokines and chemo attractants

Once, infection has occurs, the earliest response to infection is the infected cells able to release the signaling protein molecules that use for mediated immune response. Those are cytokines, chemokines, and adhesion molecules which function as cellular messenger molecules to recruit the immune cells to the infection sites. Consequently, to escape the host immune surveillance, HPV has a mechanism that can interferes the expression of cytokines pattern. One example is a study of the regulation of Tumor necrosis factor-α (TNF-α), a cytokine that induces apoptosis in number of cell types and stimulates the cytotoxic T cells to eliminate virus infected cells. This study showed that normal diploid fibroblast cell that expresses the HPV16 E7

oncoprotein has tolerance to undergo apoptosis in response to TNF-α treatment by interfering with the caspase activation [89]. The other cytokine study, is the monocyte chemoattractant protein-1 (MCP-1), the chemokine that plays an important role in the recruitment of many cell types such as monocytes, memory T cells and NK cells in solid tumors. The observation of the MCP-1 in precancerous lesions by *in situ* hybridization indicated that MCP-1 was less expreesion in CIN-1 and 2 and completely absent in CIN-3[90]. This indicate that dysregulation of MCP-1-gene expression may represent an important step during HPV-linked carcinogenesis, allowing the escape of virus-positive cells from local immune response. Interleukin-18 (IL-18) is another cytokine that has an experiment showed that its expression was interfered by HPV oncoproteins. IL-18 is a proinflammatory cytokine defined by its ability to stimulate the expression of genes associated with inflammation and plays important role in induction of IFN-γ in NK cells [91]. HPV E6 and E7 oncoproteins have ability to reduce expression and binding property of IL-18 to membrane receptor hence, loss induction of IFN-γ expression in primary peripheral blood mononuclear cell (PBMC) [92]. Thus, interfering of IL-18 expression by HPV oncoproteins may impede innate immune response especially NK cells.

Changing of the cytokine profiles

Helper T-cells (Th) can be divided into two subpopulations, Th-1 and Th-2 by the deferent of cytokine production. Th-1 cell mainly produces IL-2 and IFN-γ, while Th-2 cell produces IL-4, IL-5, IL-6, IL-10 and IL-13. Th-1 cells responsible for CMI responses, they are essential for controlling such intracellular pathogens as viruses and certain bacteria, e.g., *Mycobacterium tuberculosi*s. Whereas Th-2 provides help for

B cells which are essential for antibody-mediated immunity and allergic responses. The differentiation pathway for Th-1 and Th-2 cell development can be influenced by a number of factors, this including different cytokine stimulation patterns [93]. IL-12 is the mainly cytokine that drives the naïve $CD4^+$ T cells shift to be Th-1, whereas IL-4 is the main cytokine that drives the naïve $CD4^+$ T cells differentiate to be Th-2 [94]. Changing of cytokine profiles cause an inappropriate immune response, which may have immunosuppressive effects that result in the inability of the host to clear infection. As noted that, CMI response plays an important role in controlling of HPV infection. High grade cervical cancer (CIN-3) and cervical cancer associated high risk HPV infection flavor to produce the Th-2 cytokines when compared to low grade lesion and this correlate with progression to invasive cervical cancer [95]. Moreover, cell population of tumor draining lymph nodes (TDLN) in cervical cancer patient were regulatory T cells (T-reg) subpopulation [96]. This implied that T-reg may suppress T-cells function in cancer patients.

Modulation of adhesion molecules

Most of cells are decorated with several types of surface membrane proteins that allow it to binding to other cells or extracellular matrix. These molecules called cell adhesion molecules (CAMs). In the cervical epithelium keratinocytes, the E-cadherin, a family of hemophilic and calcium dependent CAM was demonstrated. That is a specific adhesion molecule to recruit the LC to keratinocytes [97]. As those migrations of LC depend on the control of CAM, cytokines and chemokines that expressed from keratinocytes. Some clinical observations indicated that the number of E-cadherin was significantly reduced at the site of HPV infection [98]. Moreover, down regulation of

E-cadherin was resulted by interfering of HPV E6 oncoprotien [99]. Thus, HPV E6 oncoprotein suppresses expression of E-cadherin in keratinocytes may indirectly inhibit presentation of viral antigens by LC to the immune system.

As mentioned about the immune evasion mechanism, HPV is not complicated virus and requires only a few genes to survive. But it has a grate powerful to escape the host immune surveillance. Although many details in escape mechanisms are not well understood, many immune-competent individuals who infected with HPV virus are able to clear viral infection. The persistent of high risk HPV infection are the grate risk to development of high grade and cervical cancer, but indeed progression to high grade and cervical cancer requires more than just HPV persistent. Thus, the relationship between the host immune response and HPV are needed to explain how HPV can escape immune system. This knowledge will be useful for better cervical cancer treatment strategies.

Problem in cervical cancer treatment

Although current standard therapeutic strategies for cervical cancer including surgery, radiotherapy or chemotherapy are usually effective for achieving initial disease control, management of patient with recurrence and metastatic disease is still unsolved. The recurrent rate of cervical cancer is between 10- 20% for stage Ib to IIa, clinical lesions grater than 4 cm in size and increase up to 50-70% in stage IIb to Iva (see in Appendix C) [100]. Patients with cervical cancer may develop pelvic recurrence, metastases, or combination of both. Curative treatment of recurrent patient is very difficult and rarely successful [24]. It seems to be that standard methods for

treatment cervical cancer would not be sufficient for saving patient. Therefore, the new candidate of treatment needs to be explored. One way that many researchers are trying to do is the development of immune-based therapeutic strategies.

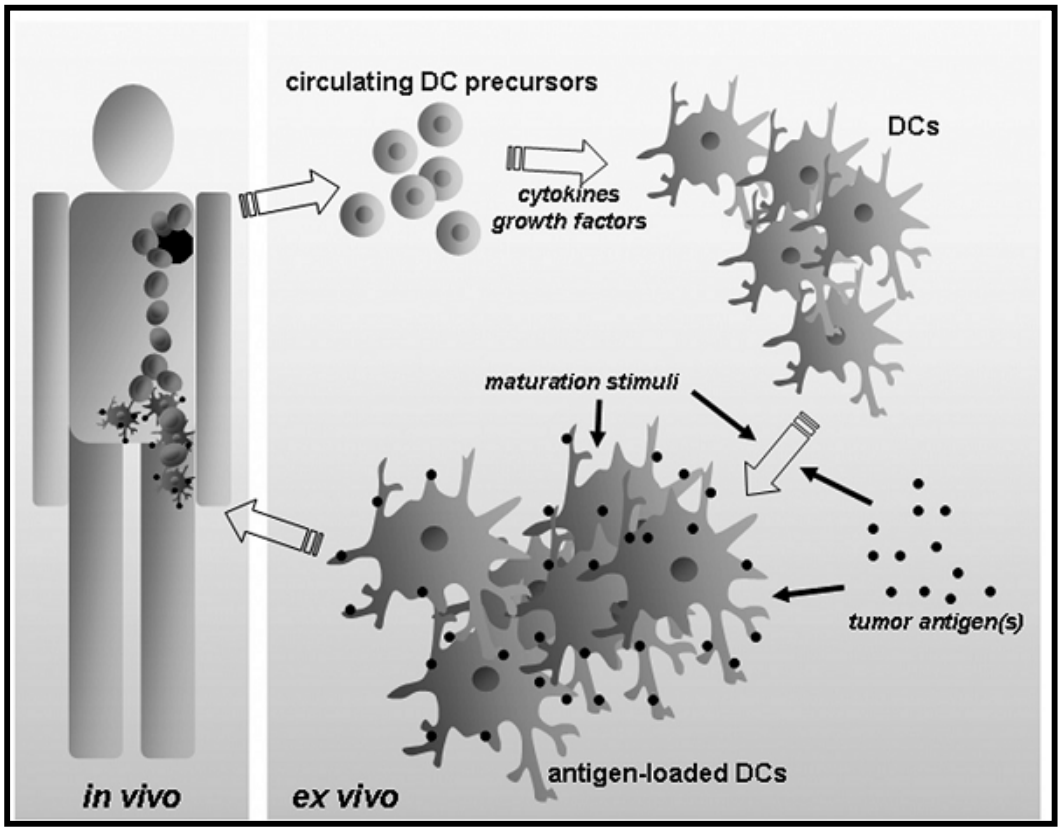
Cervical cancer immunotherapy

Recently, progression of knowledge and understanding of tumor immunology have been increased. This led to research and development methods for cancer treatment for many cancers called immunotherapies. Immunotherapies are promising treatment options for patients with many cancers. Their development is based on the concept that immune cells or their products, such as antibodies, can be engineered to recognize and kill cancer cells. Monoclonal antibody therapy, donor lymphocyte infusion, vaccine therapy and immune cell-stimulating cytokine therapy are types of immunotherapies that are in use or under study to determine their effectiveness in fighting cancers.

The view that the immunotherapy might be exploited for the treatment of cervical cancer is not new. Interestingly to date the overwhelming majority of reports in this regard have focused on the adaptive immune response to cervical cancer with specific HPV antigens. By this principle, many studies are attempted to induce HPV specific α/β T cells ($CD4^+$ and $CD8^+$) by using dendritic cells pulsed with HPV specific antigens, this called “dendritic cell vaccine”. The diagrammatic of dendritic vaccine are showed in figure 5.

Dendritic cells are recognized as a pivotal group of potent antigen-presenting cells with the ability to stimulate naïve resting T cells and to initiate primary immune responses to many viruses, microbial infections and tumors [101]. Adoptively transferred, *in vitro* manipulated DCs presenting antigen derived from different viruses have been shown to elicit CTL and Th cell responses and to induce protective antiviral immunity [102]. Recently, several groups reported the successful use of DCs for inducing antitumor immune responses in both animals and humans. [103-111]. There are many researches attempt to use DC vaccine in cervical cancer but most of them still be *in vitro* or just in phase I clinical trial [25, 112-114]. As noted above, almost of cervical cancer associate with high risk HPV infection and this virus has many mechanisms to escape the host immune response. Most of DC vaccine used specific oncoproteins E6 and E7 as antigens to activate immune cells but E6 and E7 oncoproteins usually located with in nucleus and rarely expressed on cell surface. Thus, although E6 and E7 specific T-cells clone can be generated by DC cells, this specific T cells clone may not react and kill the cancer cells. This confirmed by *in vitro* study with recombinant protein-pulsed DCs, indicating that autologous monocyte-derived DCs loaded with recombinant HPV16 E7 or HPV18 E7 can generate specific T cells response to E7 oncoproteins more than 80% in healthy individuals [115]. However, one of the arguments that this study did not observe is the killing ability of generated T cell clones to tumor cells and whether those T cell clones able to infiltrate into tumor site. Another experiment using antigen coupled to cholera toxin in combination with HPV E7 can also generate T cells specific clones. This study also showed that generated specific T cell clones can reduce HPV E6 and E7 expressing tumor cell line in mice model [114]. However, one question is that using cell line that expressed HPV E6 and E7 on cell surface that normally rarely found in cervical cancer cells may not be a good

representative, suggested that it may not happen in clinical trial. In addition, a difficulty associated with this approach is that the immunogenic of a given peptide is genetically determined by the class I alleles present in a given individual, which means that only some individuals will respond to such an immunogenic peptide. Moreover, small peptides pulse on DC are often quite unstable *in vivo* [116].



Advanced Drug Delivery Reviews (2008) [117].

Figure 5. Principle of dendritic cell vaccine. Dendritic cells, an antigen presenting cell, are harvested from a patient. These cells are then either pulsed with an antigen. The activated dendritic cells are then placed back into the patient; these cells then present the antigens to effector lymphocytes (CD4+ T cells, CD8+ T cells, and in specialized dendritic cells, B cells also). This initiates a cytotoxic response to occur against these antigens and anything that may present these antigens. One use for this therapy is in cancer immunotherapy. Tumor antigens are presented to dendritic cells, which cause the immune system to target these antigens, which are often expressed on cancerous cells.

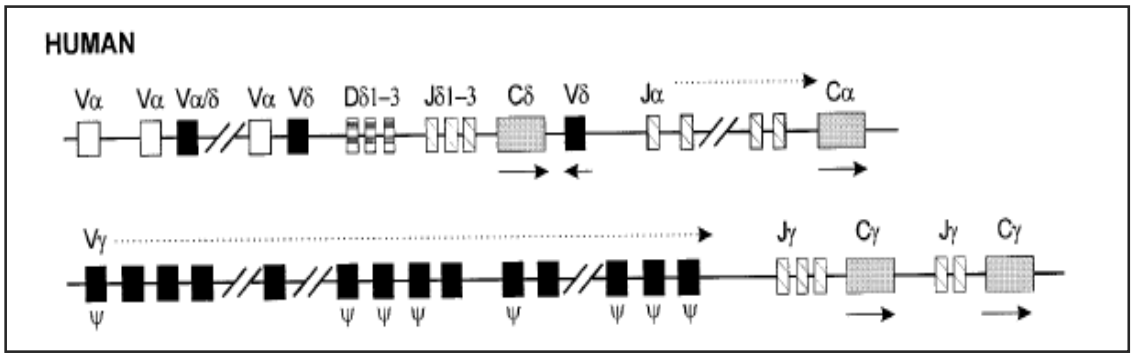
New concepts in cervical cancer immunotherapy

In order to resolve the limitation of α/β T- cells immunotherapy recently, several studies focused on gamma-delta (γ/δ) T cells. Gamma-delta T-cell was grouped in innate immune system[118]. These cells can act as immunological effector cells against several tumor cells [32, 37, 119].

Gamma-delta (γ/δ) T cell

γ/δ T cells represent a small subset of T cells (2-10% of T cell population) that possess a distinct T cell receptor (TCR) on their surface. Majority of T cells has a TCR composed of two glycoprotein chains called α- and β- TCR chains. The other hands, γ/δ T cells, the TCR is made up of one γ-chain and one δ-chain. Unlikely to alpha-beta T cells, which are commonly found in secondary lymphoid organs and have important function in adaptive immune response, most of γ/δ T-cells usually be much less common than α/β T cells. Most of γ/δ T cells locate in the epithelium layers of tissues underlying internal and external surface of the body for example tongue, esophagus, trachea, lungs, skin and genital epithelia, where they function as a first line of defense [118]. γ/δ T cells are the cells that stand on the border between adaptive and innate immune system, because the killing function of this cells looks like NK cells, innate immune cell that make a major role in anti-infectious and anti-tumor, either by directly killing the target cell or indirectly by activating the other cells in immune cells by secretion of cytokines. However, development of γ/δ T cells looks like adaptive immune cells. γ/δ T-cell and α/β T cells arise from common progenitor cells in the

thymus. The molecular events leading to the lineage decision of developing of $CD4^-$ and $CD8^-$ thymocytes to differentiate into γ/δ T cells versus alpha-beta T cells have not been fully resolved. Similar to α/β T-cells and B-cell, γ/δ T-cells also use somatic gene rearrangement of VDJ gene to generate diversity of antigen receptors as shown in figure 6 [120]. However, the number of Vγ and Vδ gene segments is limited. Only 6 of Vγ gene segment (Vγ1, Vγ3, Vγ4, Vγ5, Vγ8, and Vγ9) can be identified and only 3 Vδ genes (Vδ1, Vδ2, and Vδ3) are revealed [118, 121].



Annual Review Immunology 2000 [122].

Figure 6. Schematic representations of human TCR γ/δ gene families. TCR-V gene segments are shown as solid boxes; -D as striped boxes; -J as hatched boxes; and -C as stippled boxes. TCR gene segments indicate pseudogenes.

Human γ/δ T cells can be divided in to Vδ1, Vδ2 and Vδ3 subgroups which can be distinguished by monoclonal antibodies. Whereas most thymic or splenic γ/δ T cells expressed Vδ1 or Vδ3 TCR, the majority of γ/δ T cells (approximately 90%) in peripheral blood lymphocytes in human express TCR composted of Vδ2 and Vγ9 regions (some reports referred as Vδ2 and Vγ2) [28, 123].

Role of γ/δ T cells in microbial infection

The immunological function of Vγ9/Vδ2 T cells in humans is gradually unraveling; these cells were shown to be involved in host defense against many bacterial parasitic and viral infections. For example in bacterial infection, γ/δ deficient mice model were infected with *S. pneumoniae* lead to severe infection and all die within 7 days but this is not harmful in the wild type mice [124]. In addition, the number of neutrophils accumulation in deficient mice was less than those in wild type mice. These results suggested that γ/δ T cells played an important role in neutrophil accumulation in the lungs after infection with *S. pneumonia*, some observation was demonstrated in protozoa infection when the double γ/δ TCR knockout mice model were infected with *Plasmodium chabaudi*, the early parasitemia following the peak of infection was exacerbated, and the chronic parasitemia was maintained at significantly higher level in the γ/δ TCR knockout mice [125]. Observations of the role of γ/δ T cells in viral infection have been done especially in HIV infection. Most studies showed the number of Vγ9Vδ2 T cells associated with HIV progression. *In vivo*, HIV infected individuals showed decreased Vγ9Vδ2 T cell number, its function and inversion of the Vδ1:Vδ2 T cell ratio, with a predominance of the Vδ1 subset [126-130]. Another observation also showed that Vδ1:Vδ2 T cell ratio and impaired IFN-γ production were more evident in HIV-infected subjects with opportunistic infections than in asymptomatic patients [131].

Role of γ/δ T cells to cancer cells

The role of the γ/δ to prevent and eliminate cancer cells has now become interesting. Several observations demonstrated that Vγ9/Vδ2 T cells play an important role in tumor immune surveillance. In the beginning, most studies focus on the role of Vγ9/Vδ2 T cells to lymphoid malignancies and several *in vitro* and *in vivo* observations indicated that Vγ9/Vδ2 T cell has ability to kill many lymphoma and myeloma cells [28, 31, 38]. Furthermore, Vγ9/Vδ2 TCR gene transfer and anti γ/δ TCR antibody blocking experiments indicated that the killing of lymphoma cells was resulted by a specific Vγ9/Vδ2 TCR mediated stimulation [132]. Another aspect documented that γ/δ T cells play a necessary role in tumor immunity through provision of an early source of INF-γ that in turn may regulate the function of tumor-triggered α/β T cells. In the γ/δ T cells deficient mice had a significantly higher incidence of tumor development after challenging with a chemical carcinogen or inoculation with melanoma cell line. While in wild type mice, γ/δ T cells were recruited to the site of tumor as early as day 3 after inoculation, followed by α/β T cells at day 5 [32]. Recently, many studies are focusing on the role of Vγ9/Vδ2 T cells to many types of solid tumor. Infiltrated Vγ9/Vδ2 T cells isolated from colon carcinoma patient have ability to kill both autologous colon carcinoma cells and many colon carcinoma cell lines except normal colon cell line. In this experiment, non specific killing to many colon carcinoma cell lines imply that this killing do not depend on MHC molecule. The same experiment also showed that Vγ9/Vδ2 T cell needed T-cell receptor to recognize of IPP antigens that present on tumor cell surface[33]. Another observation revealed that tumor infiltrating γ/δ T cells isolated from individuals lung cancer patients have ability to proliferated well in culture with IL-2 and served as good effectors in killing fresh lung cancer cells [133].

Antigen for Vγ9/Vδ2 T cells

Although Vγ9/Vδ2 T cells play role in many microbial infections the specific antigens for Vγ9/Vδ2 T cell is still unclear. However Vγ9/Vδ2 T cells seems not responding to peptide antigens that present by MHC class I and II [134]. Later, Vγ9/Vδ2 T cells can recognize and respond to self and non-self non-peptide antigens, identified as small microbial compound (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a natural intermediate of the non-mevalonate pathway called MEP pathway (Figure 7) found in microbes [35, 135]. HMB-PP is an essential metabolite in most pathogenic bacteria including *Mycobacterium tuberculosis* and malaria parasites, but is absent from the human host [136]. However, in mammalian cells, another pathway called mevalonate pathway that generates the isopentenyl pyrophosphate (IPP), the intermediate products which activate Vγ9/Vδ2 T cell [35]. Interestingly, certain tumor cells produce higher concentration of IPP, can be recognized by the TCR of γ/δ T-cells as a tumor antigen[137]. Up regulation of stress ligands (such as NKG2D ligands) on infected cells and tumor cells might further enhance specific recognition of Vγ9/Vδ2 T cells. Recent studies indicated the important role played by NKG2D receptors during target cell killing by Vγ9/Vδ2 T cells [138, 139].

IPP is an intermediate in the classical HMG-CoA reductase pathway used by organisms in the biosynthesis of terpenes and terpenoids. IPP is formed from acetyl-CoA via mevalonic acid. IPP can then be isomerized to dimethylallyl pyrophosphate by the enzyme isopentenyl pyrophosphate isomerase [136] (Figure7).

Molecular basis for Vγ9Vδ2 TCR recognition of non-peptide antigens

The Vγ9Vδ2 T cells responses to prenyl pyrophosphate antigens have been clarified by cultured and stimulated *in vitro* of Vγ9Vδ2 T cells with prenyl pyrophosphate. A large expansions of γ/δ T cells can be observed by screening large panels of γ/δ T-cell clones that express a variety of V genes [140]. Moreover, antibodies to the γδ TCR blocked recognition of prenyl pyrophosphate antigens [140]. Another experiment that proved the specificity of γ/δ TCR to prenyl pyrophosphate antigens was done by transferring of γ/δ TCR of Mycobacterial reactive Vγ9Vδ2 TCR to Jurkat tumor cell line. Transfection of the Vγ9Vδ2 TCR conferred reactivity to the recipient tumor cell line to isopentenyl pyrophosphate [135]. This suggests that specific recognition of non-peptide antigens is conferred by Vγ9Vδ2 TCR expression. Moreover, the molecular basis of TCR that response to non-peptide anigens has also studied. Analysis of the CDR3 regions of Vγ9 chain has shown to be is critical part in determining of Vγ9Vδ2 TCR reactivity to non-peptide antigens. Changing of this region results in altered or abolished non-peptide antigens recognition by Vγ9Vδ2 T cells [141]. The molecules that involve in non-peptide antigens presentation is not known yet but, it is expected that non-peptide antigens can be presented by a novel extracellular pathway that does not require antigen uptake or processing [142]. The recognition and stimulation requires cell-cell contact [143]. Furthermore, MHC class I and II, CD 1a, 1b and 1c were not required for non-peptide antigens presentation [33, 142].

Mechanism of tumor cell death

Presently, the anti tumor effect excreted by γ/δ T cells to target cells has become more understanding. The important killing mechanism to tumor cells is promoting the tumor cells to undergo program cell death or called apoptosis. This mechanism can be triggered by bridging of the death receptors and ligand or trigger serine-protease enzyme called granzyme [144].

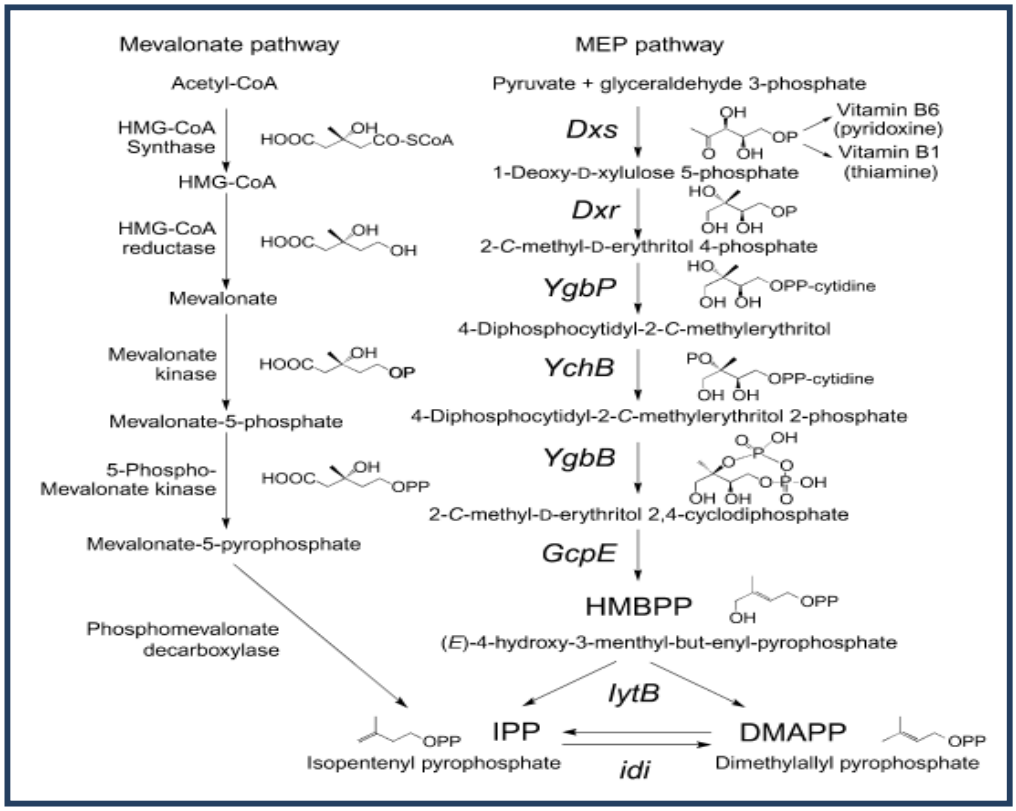
The prototypical death receptor is Fas, which upon engagement by Fas ligand (FasL) triggers the caspase cascade through its cytoplasmic domain and ultimately leads to DNA fragmentation, nuclear blebbing and cell shrinkin. In turn, the granules of cytotoxic lymphocytes contain two membrane-perturbing proteins: perforin and granzymes, all are able to activate a cascade of events leading to caspase independent cell death. A diagrammatic of apoptosis mediated by two signals is showed in figure 8.

Killing by γ/δ T cells can be mediated by both perforin and Fas/FasL pathway. Study in the role of γ/δ T cells in metastatic renal cell carcinoma indicated that the lytic activity of γ/δ T cells involves in the perforin and granzyme pathway and mainly TCR and NKG2D receptor dependent [139]. While other study showed that killing mechanism of γ/δ T cells varied depending on the mode of target cell sensitization. In those study, zolendronate, one of the nBP drug and the chemotherapeutic agents, cisplatin, were use for sensitized various type of solid tumor cell lines prior to treat with γ/δ T cells. The results revealed that zolendronate sensitized tumor cells was killed by γ/δ T cells via TCR mediated and induce perforin

release, whereas in chemotherapeutic sensitized tumor cells were killed following NKG2D mediated recognition and perforin release. However, antibody against the tumor necrosis factor related apoptosis inducing ligand (TRAIL) and FasL were unable to inhibit cytotoxicity in either chemotherapy or zolendronate sensitized tumor cells [145]. This suggested that γ/δ T cells may utilize either Fas mediated and perforin based pathway. In addition γ/δ T cells might collaborate the development of an immune response through the secretion of chemokines and cytokines such as IFN-γ, TNF-α, MIP-1-α, β, RANTES and IL8 [146, 147]. This chemokines and cytokines are functioning as chemo-attractants for activated lymphocytes, APCs and neutorphils [34]. Moreover, chemokines production by target cells are able to induce cytokines released by activated γ/δ T cells activates or migrates to target site. This according to the study in *in vitro* model of colon cancer, revealed that γ/δ T cells expressed chemokine receptors CCR3 and CXCR3 and migrated *in vitro* to chemokines secreted by colon cancer cell line (HT29 cell)

The amount of IFN-γ and TNF-α secreted by γ/δ T cells is increased when anti-tumor activity is elicited upon stimulation with tumor antigens and non-peptide antigens. The functions of both cytokines are well known, IFN-γ activates mononuclear cells, allowing these cells to performed many other functions required to kill target cell. The other important biology properties of this IFN-γ are stimulated differentiation of lymphocytes [148]. TNF-α acts as polyclonal B cells activator, increase expression of many cell adhesion molecules, such ICAM I, which important for lymphocytes migration to the target site[149]. Therefore, these chemokines and cytokines that

released form γ/δ T cells might be orchestrate the development of an immune response to combats tumor cells.



Immunol Review (2007) [136].

Figure 7. MEP and mevalonate pathways for isoprenoid biosynthesis. The MEP pathway is found in most Eubacteria (with the notable exception of Gram-positive cocci), apicomplexan protozoa, and chloroplasts, whereas the mevalonate pathway is found in Archaebacteria, eukaryotes, and the cytoplasm of plants.

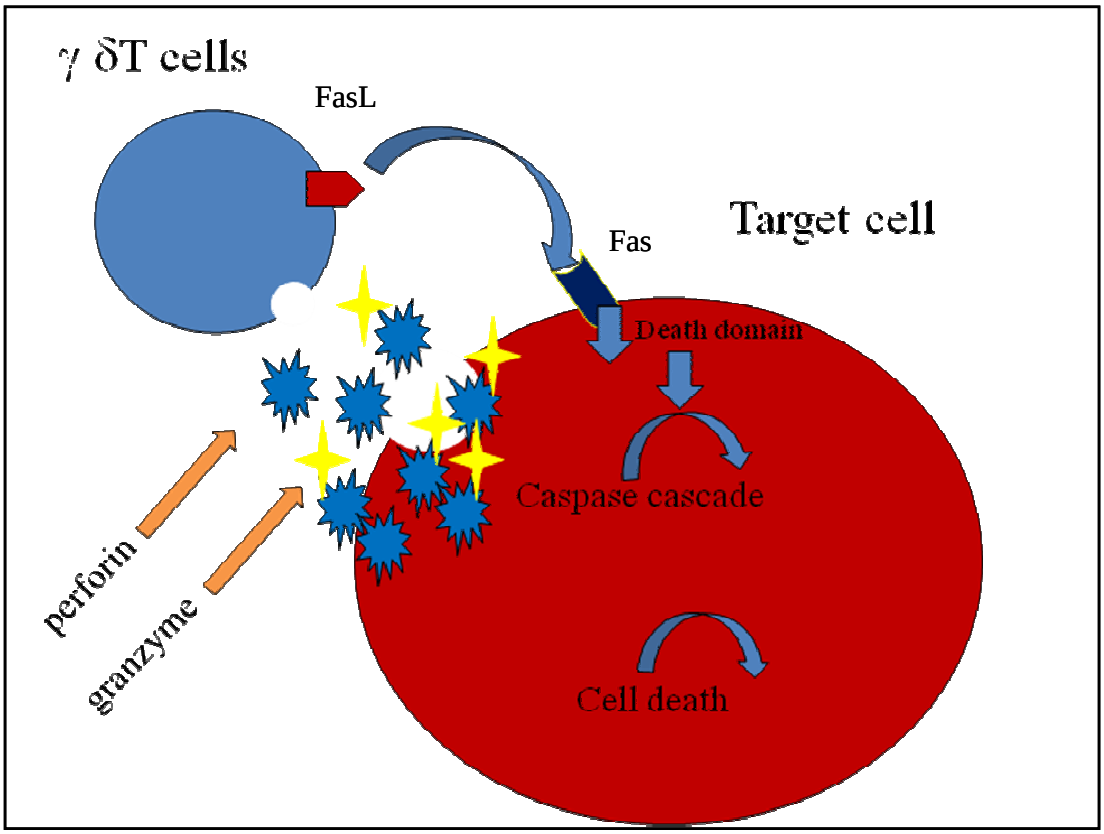
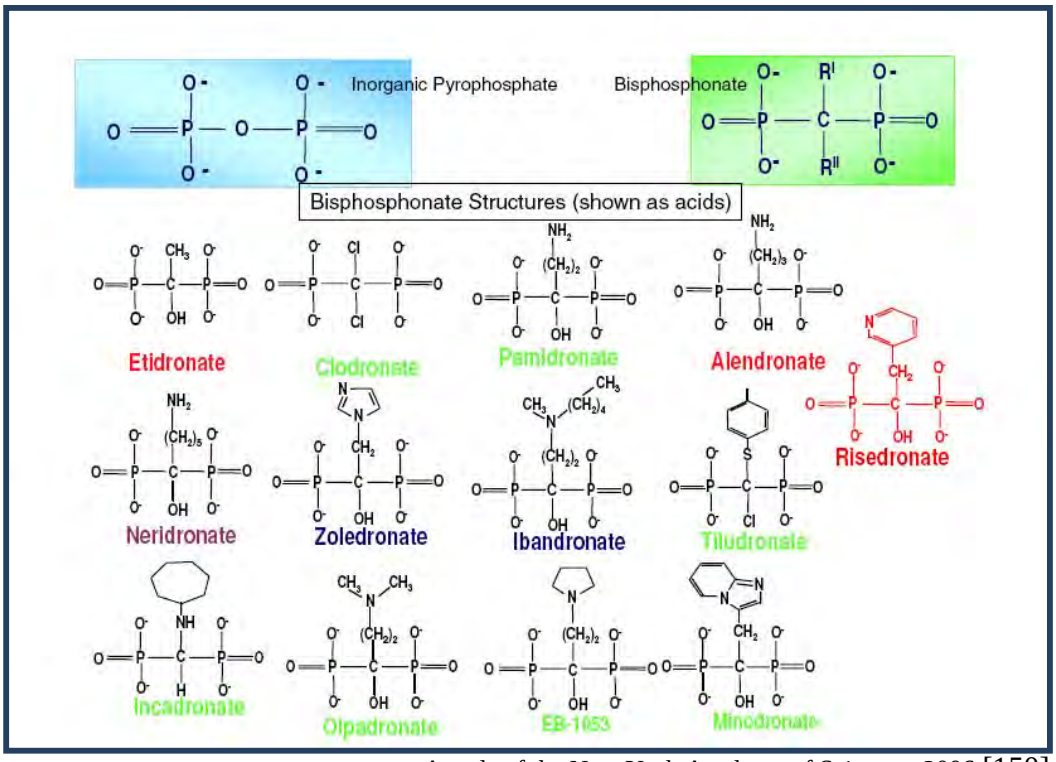


Figure 8. Apoptosis induction by Fas/FasL and granzyme-perforin pathway. Fas ligand delivery its cell death signal by binding to it receptor protein called Fas. Activation via the death domain and activate the caspase cascade resulting in cell death. Granzyme and perforin, proteins can induce apoptosis in target cells by forming transmembrane pores and through cleavage of effector caspases.

Drug induce accumulation of IPP

There are a number of drugs nitrogen bisphosphate (nBPs) that can cause accumulation of intracellular IPP such as pamidronate, Alendronate, Risedronate, Ibandronate, and Zolendronate. Nitrogen bisphosphate are the potent drug that used for inhibiting the osteoclast-mediated bone resorption and used for treatment patients with bone metastasis [37]. It was first synthesized in the 1800, during that time it was used in industry mainly in textiles, fertilizer and oil industries. The ethidronate was the first bisphosphonate that used as a medicine in 1960 [150]. Nowadays, several derivatives of nBPs have been constructed (Figure 9).

This compound induces accumulation of the IPP in the cells by inhibiting the FPP-synthase, a key enzyme at the down stream of IPP product (Figure 10). Several observations documented that use of nBP drugs do not only induce proliferation of Vγ9Vδ2 T cells but also enhance the killing property of Vγ9Vδ2 T cells to target tumor cells [35].

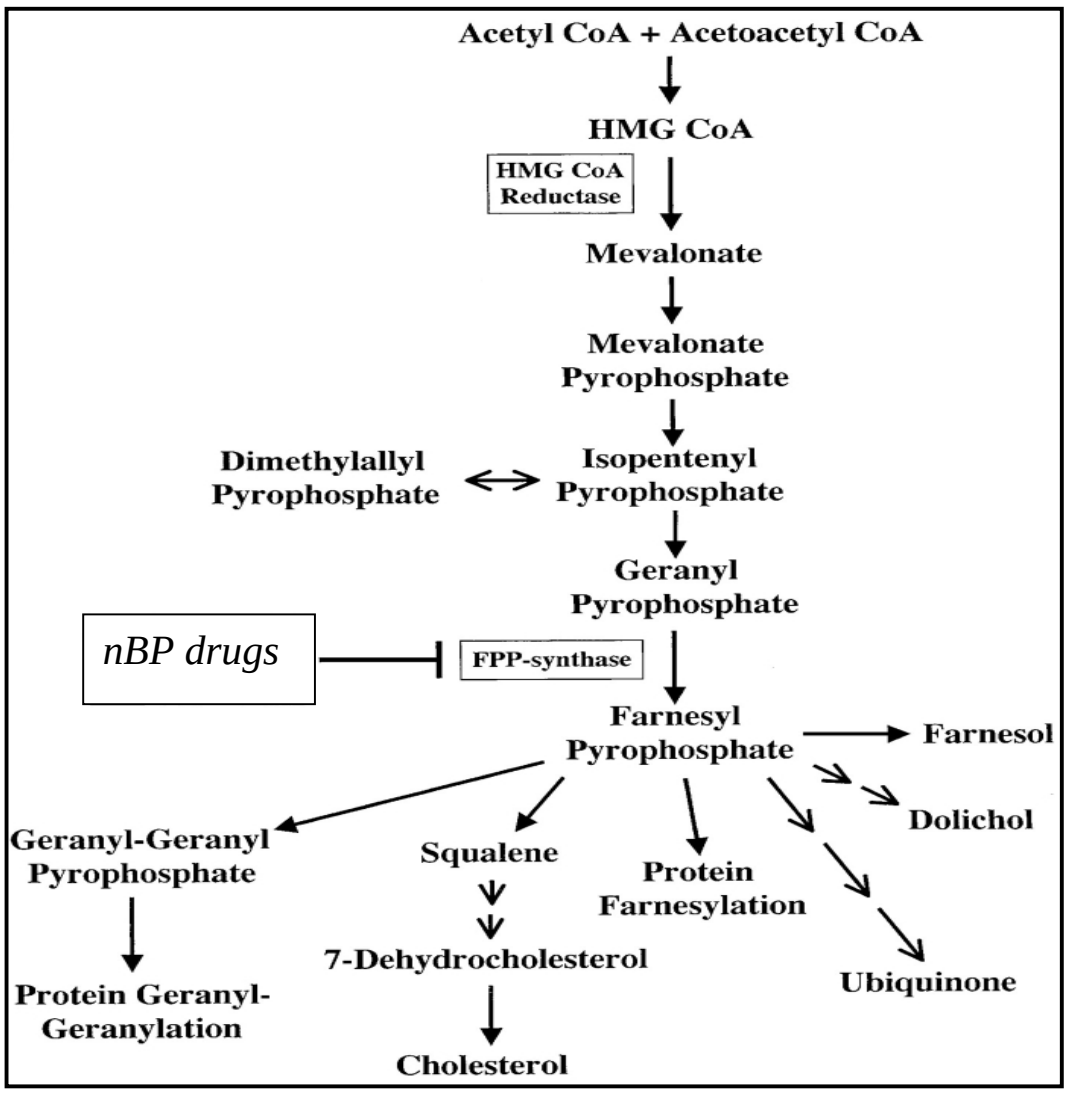


Annals of the New York Academy of Sciences. 2006 [150].

Figure 9. Structures of the bisphosphonates

Pamidronate is one of choice. In patients who received pamidronate, high level of γ/δ T cells especially Vγ9Vδ2 T cells were demonstrated in peripheral blood when compared to the level prior drug infusion [119].

Here, in our experiment, the pamidronate was selected as representative of the nBP drugs. Evaluate the induction and proliferation of Vγ9Vδ2 T cells as well as cytotoxicity to cervical cancer cell lines will be explored.



Journal of Experimental Medicine 2003[35]

Figure 10. The mevalonate pathway and mechanism of nBPs to inhibit the FPP-synthase in mammalian cells.

How to study cervical cancer

From the foregoing all the above**,** the strong association of HPV and cervical cancer and the problem for recurrent and metastasis cervical cancer were revealed. Although, several researchers attempt to explore the new methods for treatment patients with cancer stage, unfortunately at present there is no suitable *in vivo* model for studying the pathogenesis and treatment of this cancer. The major obstacle in developing an animal model is that almost 100% of cervical cancer are associated with HPV. Unlikely other viruses, propagation of HPV in the cell culture system is difficult. This is because the virus is highly species specific and its replication is regulated by the differentiation pathway of keratinocytes. Therefore, the success to development of the new approach for treatment methods such as immunotherapy, chemotherapy or vaccine trial for HPV infection has been impeded due to lack of suitable animal model for study the viral pathogenesis. Thus, the animal model system for vial interaction and cervical carcinogenesis would be very useful. This *in vivo* model would allow for studying viral pathogenesis and furthermore, may support for vaccine or any antiviral compounds evaluations.

During the past decades, many studies have been attempts to develop the animal model for study the association of HPV and cervical cancer. The early study in 1987 was developed a laboratory production of HPV 11 by infection of vulva condyloma on to human foreskin and grafted beneath the renal capsule of athymic mice. This study showed the condylomatous cysts developed from grafted tissue and the nuclei of lesion cell showed the large amount of capsid antigen and intranuclear virions [42]. However this model is not suitable for cervical cancer because the grafted

was infected with low risk HPV and the environment of the renal capsule are quite different from cervical cell. Moreover, evaluation of the therapeutics effects within renal capsule is not practical. Subsequently, transgenic mice model with E6 and E7 oncoproteins were generate to evaluate the role of such viral oncoproteins. In this study, the characteristics of cancer cells occur in transgenic mice were proved to be associated with HPV E6 and E7 oncoproteines. However, transgenic mice did not produce neoplasia or cancer [43]. However, the transgenic mice model with viral oncoproteins does not demonstrated the interaction between human tissue and the virus. Hence, using of transgenic mice model for study the role of new therapeutic agents such as new drugs, and vaccine testing, may unacceptable. Another study attempted to implant HPV-6 and/or 11 infected in meshed human foreskin on the dorsal of SCID mice. Grafting of HPV 6 and 11 resulted in the formation of a differentiated and exhibit the hallmark of features of HPV infection including basal hyperplasia, acanthosis and koilocytosis [151]. However, like the other previously studies, they do not show the association of high risk HPV and the features of cervical cancer in grafted mice. Therefore, it can not be useful as therapeutic model for treatment of cervical cancer.

The ideally animal model that can be supporting for new therapeutics for cervical cancer and HPV should breakthrough a limitation as mentioned above. The mice model should easily be able to show the beginning steps of tumor establishment such as increase tumor size, vascularization, association with HPV infection, reliable and reproducible. Therefore, in this, we also attempt to generate malignancy tumor in mice by incoculating of cervical cancer cell line on the dorsal skin of nude mice for further γ/δ T cells immunotherapeutic study.

CHAPTER III

MATERIALS AND METHODS

Part I Establishment of animal model

1.1 Cell line cultivation

Cell lines used in this study were HeLa, CaSki, SiHa cell lines and NTY cell. HeLa cells were obtained from Virology Unit, Department of Microbiology, Chulalongkorn University. CaSki and SiHa were obtained from ATCC (CRL-1550 Lot No: 3794357 and HTB-35 Lot No: 4031219 respectively). NTY cell was the normal fibroblast cell which isolated from cervical cancer patient, from department of Obstetric and Gynecology, Faculty of Medicine University. All of them were grown in MEM media (GIBCO BRL,USA.) supplemented with 10% fetal bovine serum (FBS) (PAA; Austria), 50 unit/ml and 50 ug/ml of penicillin/streptomycin (Bio Basic Inc., Canada) and 2 mM L-glutamine.

In order to maintain the cell lines in 25 cm^2 flask, the culture medium was removed from tissue culture flask and the cell was washed one time with 5 ml of 1x PBS (see Appendix B). After removing PBS, one ml of pre-warmed (37℃) of trypsin versine (see Appendix B) was added and it was incubated for 1-5 min at 37℃. After discarding trypsin, cell was detached from culture flask by sharply against on palm. After completely detached the cell, 3 ml of growth medium (see Appendix B) were added in to flask. The cell suspension was mixed by pipetting up and down. After that each 1 ml was transferred in to new 3 flasks and additional added 4 ml of MEM into

each tissue culture. The cell was incubated at 37℃ with 5% CO_2 in atmosphere. The cell was usually sub-cultured in every 4-5 days intervals.

1.2 Preparation of the cell line for implantation

To prepare the cell line for implantation, the maintained cell line from 25 cm^2 tissue culture flask was trypsinized and transferred to new 75 cm^2 tissue culture flasks with the ratio 1:1. The cell was incubated at 37℃ with 5% CO_2 in atmosphere for 3-4 days. After that, cell was harvested by trypsinization as described previously. After trypsinization, the number of cell and the percentage of cell death was determined by hematocytometer. The percentage of cell death must be less than 10%. After that, the cell was adjusted the volume with MEM to 2.5×10^5, 5×10^5, and 1×10^6 cells per 30 ul. The cell was stored in the 4 ℃ or on ice all the time during the process of implantation.

1.3 Nude mice

Female BALB/c-nude mice were purchased from National Laboratory Animal Center of Salaya Campus, Mahidol University, Bangkok, Thailand. The aged 4-8 weeks and weighed 20–25 g were used. The animals were handled as recommended by the guide for the care and use of experimental animals 1996, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, USA.

1.4 Implantation of cervical cancer in nude mice

The procedure of implantation using dorsal skin fold window chamber was followed by Yoysungnoen Y., 2004 [152]. HeLa cells, a cervical cancer cell line containing HPV-18 DNA integrated in the host chromosome approximately 500 copies/cell were used. The cells were maintained by culturing in MEM media as described above. The ability of HeLa cell to produce tumor *in vivo* was determined by implanting of various doses of live cells. Control mice received only MEM medium. On the day of implantation, mice were anesthetized with pentobarbital sodium 40 mg/kg by intraperitoneal (IP) injection. After anesthetization, the dorsal skin was removed and the dorsal- skinfold chamber was fixed on the dorsal skin. The HeLa cell at various concentrations (2.5×10^5, 5×10^5, and 1×10^6 cells) was implanted into the dorsal skinfold chamber (Figure 11).

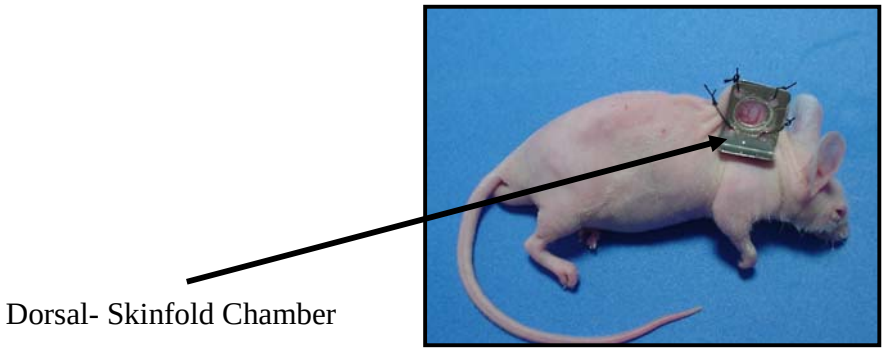


Figure 11. Method for implantation of HeLa cell in to mice. Skin fold window chamber was fixed on the dorsal skin of mice (arrow)

1.3 Tumorigenesis confirmation

In order to evaluate tumor that grows in nude mice, implantation mice were tested for angiogenesis, histopathology changes, PCR for detection of HeLa DNA and *in situ* hybridization with probe specific to HPV-18.

1.3.1 Intravital fluorescence videomicroscopy

For the study of tumor angiogenesis, intravital fluorescence videomicroscopy was used. Two weeks after 1×10^6 HeLa-inoculation, the mice were anesthesized with an IP injection of sodium pentobarbitol (50 mg/kg). A catheter was inserted into a jugular vein for an application of fluorescence tracers [FITC-dextran, MW = 200,000, 0.5% (Sigma Chemical, USA)]. The tumor microvasculature was observed under an intravital fluorescence microscope (Nikon, Japan) equipped with a videocamera (Sony SIT68, Japan) and video-recorder (Sony SVT-124P, Japan). The 10× objective lens was used to observe microvessels within the tumor-bearing chamber. Based on the recorded videoimages, the tumor microvascular networks including neocapillary density were analyzed by using digital image processing software (Global Lab II).

1.3.2 Tumor size, tumor weight and histopathological examination

Tumor size was assessed using vernier calipers (VWR, St. Louis, USA). After the mice were anesthesized with an IP injection of sodium pentobarbitol (50 mg/kg). Tumor mass was carefully collected immediately, weighed and fixed into neutral buffer formalin (see Appendix B) when euthanasia the mice with over dose of pentobarbital sodium. Formalin-fixed, paraffin-embedded tissue samples were cut in 5 µm thick sections on a microtome with a disposable blade and observed under microscopy after Hematoxylin and Eosin (H&E) stain.

1.3.3 HPV-DNA detection

The fresh tumor tissue was extracted by using Qiagen DNA extraction kit (Qiagen, USA). The procedure was performed according to recommendation of the company. In brief, approximately 20 mg of tumor tissue was used. Tissue was cut in to small pieces then, placed it into 1.5 ml microcentrifuge tube. After that, 20 ul of proteinase K (provide by company) was added, and incubated at 56℃ until tissue was completely lysis. After that, 200 ul of AL buffer was added and additional incubated at70℃ for 10 min followed by adding 200 ul of absolute ethanol, and gentle mixed for 15 sec. The mixture sample was then applied into QAI spin column and centrifuged at 8,000 rpm for one min. Then the QIAamp spin column was sequentially washed twice by centrifugation at 8,000 rpm with 500 ul of AW1 buffer. The sample was then washed once by centrifugation at 14,000 rpm with 500 ul of AW2 buffer. The sample DNA was eluted from the spin column after by adding 200 ul of AE buffer and

incubated at room temperature for 1 min before centrifugation at 8,000 rpm. The eluted DNA was stored at -80℃ until used.

The extracted DNA was determined for the presence of HPV genome by PCR using specific HPV-L1 primers. All primers were purchased from Invitrogen. The primers sequence and reaction conditions were shown in table 1 and 2**.**

Table 1. Properties of oligonucleotides primers

Name	Sequence (5'- 3')	Target
L1C1	CGTAAACGTTTTCCCTATTTTTTT	HPV-L1
L1C2	TACCCTAAATACTCTGTATTG	HPV-L1
PC04	CAACTTCATCCACGTTCACC	Human β goblin
GH20	GAAGAGCCAAGGACAGGTAC	Human β goblin

Table 2. Condition of PCR for each primers

Primer	reaction (50 ul)	condition	product size
L1C1 **L1C2**	1X buffer (100 mM. KCl, 20 mM Tris) 1.5 mM $MgCl_2$ 200 uM dNTPs 25 pmole primers 1.25 units of Taq 1 ul of DNA sample	Start 95 ^{O} C 10 min 1 cycle Denature 95 ^{O} C 1.30 min Annealing 40 ^{O} C 1.30 min Extension 72^{O} C 2 min Extension 72^{O} C 10 min 40 cycels	250 bps.
PC04 **GH20**	1X buffer (100 mM. KCl, 20 mM . Tris.) 1.5 mM $MgCl_2$ 200 uM dNTPs 2.5 pmole primers 1.25 units of Taq 1 ul of DNA sample	Start 94 ^{O} C 10 min 1 cycle Denature 94 ^{O} C 30 sec Annealing 62 ^{O} C 1min Extension 68^{O} C 1 min Extension 68^{O} C 1 min 40 cycles	268 bps

1.3.4 HPV genotyping

DNA extracted from tumor cells was analyzed for HPV genotyping by using HPV genotyping kit (Inno-Lipa HPV Genotyping V2; Innogenetics; Germany). The principle of this technique is based on the reverse hybridization. A part of L1 region of the HPV genome was amplified and denatured biotinylated amplicons were hybridized with specific oligonucleotide probes which were immobilized as parallel lines on membrane strips. The procedure of test was performed as recommendation by company. In brief, firstly 10 ul of denaturation solution was added into each chamber followed by 10 ul of amplified product, the mixture was incubated at 20-25℃ for 5 min. After that 2 ml of pre warmed hybridization buffer was added into the sample. The test

strip was then put into the mixture of hybridize solution and incubated at 49℃ in shaking water bath for 1 hr. After, the test strip was washed with 2 ml of stringent wash buffer and incubated at 49℃ in shaking water bath for 30 min. After that, strip was washed with rinse solution (the rinse solution and conjugate was prepared according to the manual) for one min at 20-25℃ on a shaker. After that 2 ml of conjugate solution was added into each strip and incubated at 20-25℃ for 30 min on a shaker, again the strip was washed twice with 2 ml of rinse solution at 20-25℃ for one min on shaker, once with 2 ml of substrate buffer and incubated at 20-25℃ for 30 min. Finally, 2 ml of substrate solution was then added into each strip and incubated at 20-25℃ for 30 min. The strip was then rinsed in distilled water for 3 min.

1.3.5 *In situ* hybridization of HPV-DNA

The presence of HPV-DNA in cells was detected by *in situ* hybridization using a probe specific to HPV-16/18 obtained from the Enzo Diagnostics kit (USA) in combination with the Dako GenPoint kit (Denmark). The system is colorimetric detection using tyramide reporter molecule to amplify the number of biotin which enhances binding of streptavidin-horseradish peroxidase (HRP) complex and 3,3′ diaminobenzidine (DAB) was used as substrate. Then, the tissue was counterstained with Hematoxilin. The protocol was performed according to the manufacturer's recommendation. This assay was kindly performed by Professor Dr. Wasun Chantratita, Division of Molecular Virology, Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand.

Part II *In vitro* study: the cytotoxicity of γ/δ T cells

2.1 PBMC Isolation and cultivation

PBMC were derived from buffy coats of healthy adult blood donors from Thai Red Cross blood bank. PBMC was isolated by Ficoll-Hypaque density gradient centrifugation. In brief, PBMC from blood dornor was diluted 1:1 with serum free RPMI 1640 medium (PAA,Austria). The 40 ml of diluted blood was overlaid on 10 ml of Ficoll-hypaque in 50 ml centrifuge tube. The overlaid blood was then centrifuged at 2,800 rpm at 20℃ for 30 min. The new buffy coat layer was then collected into a new 50 ml tube. The buffy coat was washed with citrate PBS buffer (see Appendix B) several times until the supernatant was clear by centrifugation at 1,500 rpm at 20℃ for 10 min. After that PBMC was finally washed with serum free RPMI 1640. The PBMC was counted using hemacytometer and determine the cell viability with 0.4% trypan blue. Approximately 1×10^7 cells were then seeded in to 75 cm^2 tissue culture flask.

Cultivation of PBMC and activation of γ/δ T cell was modified form Salot S., *et al*, [153]. PBMC were cultured in RPMI-10 medium (see Appendix B) and incubated at 37℃ in humidified atmosphere of 5% CO_2 condition. PBMC were stimulated with single dose (10 uM) of pamidronate (Novartis) at the first day of culture together with 100 U/ml of IL-2 (Pepro Tech; USA). To establish Vδ2/Vγ9 T cells, PBMC was re-stimulated with IL-2 100 U/ml at 3 days interval.

2.2 γ/δ T cells purification and cultivation

At 14 days of PBMC cultivation, the γ/δ T cells were harvested and purified from PBMC by using specific monoclonal antibody (mAb) to γ/δ TCR (miniMACS; Miltenyi Biotech, Germany). The procedure was followed by company's instruction. PBMC was collected by centrifugation at 300×g, 4℃ for 10 min. Approximately 10^7 of PBMCs were then re-suspended with 80 ul of MACS buffer (PBS pH7.2 with 0.5% bovine serum albumin, 2 mM EDTA) and 20 ul of biotin-antibody was added. Then cells were incubated at 4℃ for 10 min. After that the cells were washed with 1-2 ml of buffer by centrifugation 300×g, 4℃ for 10 min. After removing the solution 80 ul of the MACS buffer and 20 ul of anti-biotin microbead were added. The cells were further incubated at 4℃ for 15 min, and washed again with 1-2 ml of MACS buffer by centrifugation 300×g at 4℃ for 10 min. After aspirate supernatant completely, the cells were re-suspended with 500 ul of MACS buffer and were applied into MACS column. During the cells were passing through the column, the column was washed with appropriated amount of MACS buffer. The cells that retained within the column were γ/δ T cells. The γ/δ T cells were collected by using column plunger propel the column; the cells that pass through this propelling were γ/δ T cells.

Purified cells were cultured in RPMI-10 (see Appendix B) and supplemented with 100 U/ml of IL-2 for 3 days before determining cytotoxicity assay. The cell was counted using hemocytometer and determined the cell viability with 0.4% trypan blue. The purity of cells was confirmed by flow cytometry using specific mAb to Vδ2/Vγ9 TCR (BD Bioscience; USA).

2.3 Proliferation assay

A crystal violet assay was used to determine cervical cancer cell proliferation was followed by Senaratne, S.G., *et al* [154]. In brief, approximately 1×10^4 of cervical cancer cell lines (HeLa, SiHa, and CaSki) were cultured in 48 wells plate. The cells were cultured in MEM supplemented with various doses of various concentration of pamidronate (0, 1, 10, 20, 40, 80, 100 and 200 uM/ml). After that the cells were incubated in 37℃ in 5% CO_2 in atmosphere for 5 days. On the selected days after removal of the tissue culture medium, the cells monolayer was fixed with methanol prior to staining with 0.5 ml of 0.5% crystal violet solution in 25% methanol for 10 min. After washing several times with distilled water to remove the excess dye, the cells were air dried for at least 20 h. The incorporated dye was solubilized in 1 ml (48-well plates) of 0.1 M sodium citrate in 50% ethanol and 100 µl aliquots were transferred to 96-well plates. In order to determine cell number in each sample, the optical density (OD) was measured directly at a wavelength of 550 nm in a microplate reader (Humanreader, Human, Gesellschaft, Taunsstein, Germany). The OD of each sample was then compared with a standard curve, in which the OD was directly proportional to known cell numbers.

2.4 Cytotoxicity assay

In order to determine the cytotoxicity of γ/δ T cells to cervical cancer cell lines, the lactate dehydrogenase (LDH) cytotoxicity assay kit was used. The principle of this technique was based on measurement of LDH enzyme that released from damaged cells. Since LDH enzyme is a stable cytoplasmic enzyme present in all cells and rapidly

released into the culture medium upon damage of the plasma membrane. The LDH enzyme can be measured by using enzymatic reaction. This LDH enzyme can be oxidizes the substrate, lactate to be pyruvate which then reacts with tetrazolium salt INT to form formazan. The amount of formazan is directly correlates to the amount of LDH in the culture, which is in turn directly proportional to the number of dead or damaged cells. The formazan can be measured by using spectrophotometer at 500 nm. The procedure for LDH cytotoxicity assay kit was followed by the company's construction.

Approximately $1x10^4$ of actively proliferating HPV DNA positive cervical cancer cell lines (HeLa, CaSki,SiHa cells) were seeded in 96 well, u-shape bottomed microtiter plates. The cells were cultured in RPMI-10 with and without 10 uM of pamidronate and incubated at 37℃. After 24 h, cells were washed twice with PBS pH 7.2 and once with RPMI-10, then various numbers of effector cells (ratio Effector : Target = 1:20 and 1:40) were added into each well. Finally fresh RPMI-10 supplemented with low dose IL-2 (20 U/ml) was added into each well. Effector and target cells were subsequently co-cultured for 5 h at 37℃ in humidified atmosphere of 5% CO_2 condition. After that the supernatant from each well was collected and tested immediately for releasing of LDH from death cells by using LDH Cytotoxicity Detection Kit (Roche, USA). Preparations of all reagents kit were followed to the manufacturer's protocol. The low control in the system was target cells plus 50 ul of assay medium. The high control was target cells plus 50 ul of assay medium and 5 ul of lysis solution was added in the last 15 min before ending of incubation. The assay medium alone was used as background control. The OD was determined directly at 492 nm and the percentage cytotoxicity was calculated by the following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{exp. value - low control}}{\text{high control - low control}} \times 100$$

2.5 Measurement of cytokines production

Approximately 50 ul of supernatants from cytotoxic assay were separately aliquoted and stored in -80℃ for further IFN-γ and TNF-α detection. The cytokines detection were used commercial available ELISA detection kit (PeproTech, USA). The procedure was as followed.

Plate preparation

The capture antibody was diluted with PBS to a concentration of 1μg/ml and immediately added 100 μl to each ELISA plate well. The plate was sealed and incubated overnight at room temperature. Then liquid was removed from each well by aspirating and plate was washed 4 times by using 300μl of wash buffer per well. After the last washed plate was inverted to remove residual buffer and blotted on paper towel. Then 300 μl of block buffer was added to each well and incubated for at least one hour at room temperature. Then, plate was washed 4 times with washed buffer.

ELISA protocol

Standard/Sample: The standard cytokines either IFN-γ or TNF-α provided by the kit was diluted from 2 ng/ml to zero in diluent solution. Then 100 μl of standard and sample was immediately added into each well in duplicate. The plate was then incubated at room temperature for at least 2 h.

Detection: After 4 hr plate was washed 4 times with washed buffer. Then the detection antibody was diluted in diluent to a concentration of 0.25µg/ml, and 100 ul of diluted antibody was added into each well. Plate was continuously incubated at room temperature for another 2 h.

Avidin-HRP Conjugate: Plate was washed 4 times with washed buffer by aspirating. Then 5.5µl of avidin-HRP conjugate was diluted to 1:2000 with diluent for a total volume of 11ml, and 100µl of diluted conjugate was added in each well. Plate was incubated 30 min at room temperature.

ABTS Liquid Substrate: Plate was washed 4 times with washed buffer by aspirating. Then 100µl of substrate solution was added into each well. Plate was then incubated at room temperature for color development. During the incubation the plate should be protected from light. Finally, the color development was observed by ELISA plate reader (Humanreader, Human, Gesellschaft, Taunsstein, Germany) at 405 nm with wavelength correction set at 650 nm.

2.6 CD107 cytotoxicity assay

Activation and responding mechanism of T cells to various types of tumor antigen usually mediated via major two pathways, a granule-dependent (perforin/granzyme) and independent (ligand-ligand induce cell death, e.g. Fas-FasL) mechanism. The granular dependent pathway, dose not requires de novo synthesis of proteins but, associated with releasing and de-granulation of lytic granules located within cytoplasm. The lytic granules are containing a dense of protein mediators involves in cell apoptosis such as perforin and grandzyme. The core of lytic granule surrounded by lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs) including CD107 [155, 156]. Localization of CD107 on lytic granule was

proposed to prevent against the leakage of cytotoxic mediators by protecting the interior of granule membrane. A diagrammatic representation of this event is showed in figure 12.

Therefore, measurement of the mobilized CD107 on surface membrane can be used to determine the phenotypic and functional characteristics of responded T cells. Hence, this experiment, we applied this observation method to characterize the killing mechanism of γ/δ T cells to cervical cancer cell lines. The CD107 assay was followed the method described by Betts et al. [157]. Approximately 10^4 of cervical cancer cell lines (HeLa, SiHa and CaSki) were grown on 96 well plate using MEM with or without 10 uM/ml of pamidronate and incubated at 37^{o}C in 5 % CO_2 in atmosphere for 12 h. The next day before assay, the culture media was removed and cells were then washed with PBS for once. Approximately 2×10^5 of the γ/δ T cells were added in each well followed by adding 3 µl of CD107-FITC (BD Bioscience; USA). Next, the plate was the centrifuged and then incubated in CO_2 incubator at 37^{o}C for one hour prior to add 10 µg/ml of brefeldin A to each well. The plate was additional incubated for 4 h. The cells were collected in to flow cytometer tube, washed once with PBS and stained with anti-human Vγ9 TCR-PE for 15 min. The cells were washed again with PBS and finally re-suspended in 200 µl of PBS and analyzed by FACSCalibur flow cytometer (BD Bioscience; USA).

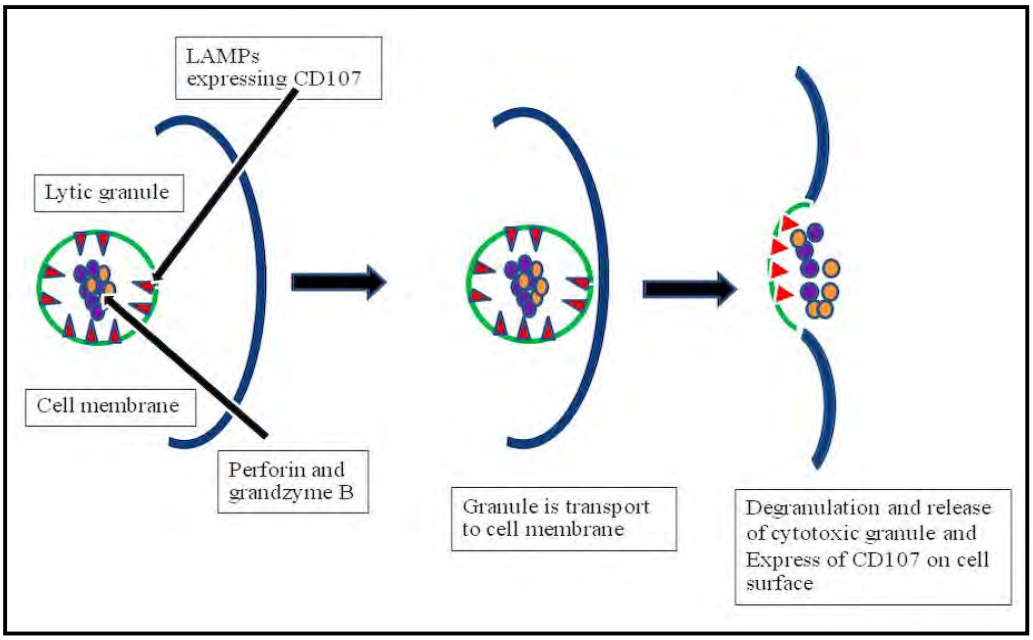


Figure 12. Diagrammatic representation of the principle underlying the CD107 degranulation assay. CD107 are expressed on the inner leaflet of granule membranes, and are thus transiently exposed on the cell surface upon granule fusion with the plasma membrane.

Part III. *In vivo* study of γ/δ T cells in mouse bearing tumor model

3.1 γ/δ T cells labeling with cell tracker dye and cells trafficking

CellTracker reagents are fluorescent chloromethyl derivatives that freely diffuse through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes react with intracellular components to produce cells that are both fluorescent and viable for at least 24 h after loading. CellTracker Green (5-chloromethylfl uorescein diacetate) CMFDA (Invitrogen; USA) has a relatively low pKa, which ensures that it will exhibit bright, green fluorescence in the cytoplasm at all physiological pH levels.

In order to label purified γ/δ T cells, the cells were collected and washed by centrifugation at 300×g for 10 min with PBS. After that, cells were washed again with serum free RPMI 1640. Then collected cells were incubated with 25 uM of CMFDA at 37℃ in CO_2 incubator with protecting exposure from light for 45 min. The labeled cells were washed again with serum free RPMI 1640 and resuspended in serum free medium RPMI 1640 and immediately use.

In order to assay the efficacy of γ/δ T cells *in vivo*, approximately 2 weeks of tumor developed mice were used. Mice were anesthetized with pentobarbital sodium (40 mg/kg; IP). After that, approximately 3×10^6 cells of the labeled γ/δ T cells with CMFDA were injected into several position on the tumor size. The control mice were injected with labeled flow though PBMC from the γ/δ purification process. Then trafficking of γ/δ T cells was observed and taken a photograph by using *in vivo* imaging

System (Kodak, USA). Then mice were lived for 3 days before scarified and collected tumors tissue for further evaluation.

3.3 Histopathology study

3.3.1 H&E staining

In order to study histopathology changes, the developed tumor mice were sacrificed by injecting over dose of pentobarbital sodium. After that, tumor tissue was cut from the mice and immediately fixed in neutral buffer formalin (see Appendix B) for 48 h. For tissue staining, approximately 5 um of paraffin embedded tissue was sliced and fixed on to slide. Tissue section was stained with H&E by using automatic tissue staining (LEICA, Autostaining XL, Germany).

3.3.2 Anti CD3 staining

To observe the deposition of γ/δ T cells within the tumor areas, tissue was stained by using *in situ* immunohistochemistry technique. Approximately 5 um of the formalin-fixed, paraffin embedded tissue was subjected to antigen retrieval by high pressure cooker treatment for 10 min. The tissue was stained with specific human anti CD-3 polyclonal antibody (Rabbit anti Human CD3 A0452, DAKO, USA). The antibody was diluted in 1:500 with citrate buffer pH. 6.0 (see Appendix B). The secondary antibody and detection was used the envision detection system (envision^{tm} system,HRP, K4003,DAKO,USA).Staining process of tissue was done by using automatic staining machine (Ventana, Benchmark LT, USA).

3.3.3 *In situ* apoptosis detection

Apoptosis is defined as a mechanism of cellular suicide or program cell death (PCD) that controls by complicated biochemical signaling pathway. Characteristic of apoptotic cells include blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation due to the controlled digestion of DNA by DNAses. The end result of apoptosis is cell death without inflammation of the surrounding tissue. Observations of apoptotic bodies characteristic are difficult, since it have a short-lived appear only in a few minutes. In contrast with DNA fragmentation which followed by cell death and removal from the tissue, usually found in several hours. Therefore, detection of DNA fragmentation should be the appropriate method for detection of apoptosis in tissues.[144] One example method that detect DNA fragmentation in apoptosis is the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker [158]. In this experiment, *in situ* apoptosis detection kit (ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit; CHEMICON International, USA) based on DNA fragmentation was used to prove apoptosis within tumor after injecting with Vγ9/Vδ2 T cells. The procedure was followed by the company's instruction.

1. Deparaffinized tissue section

Approximately 5 um of formalin-fixed paraffin-embedded tissue was sliced and fixed onto slide. After that, tissue sample was de-paraffinized by washing in 3 changes of xylene for 5 min in each wash followed by 2 changes of absolute ethanol for 5 min in each wash and finally washed with PBS for 5 min.

2. Pre-treat tissue

Twenty ug/ml of proteinase K was applied to tissue and incubated at room temperature for 15 min. After that tissue slide was washed in 2 changes of PBS in a coplin jar for 2 min in each wash.

3. Apply equilibration buffer

Tissue slide was gently tap off excess liquid around the section, then 75 ul/ 5 cm^2 of equilibration buffer was applied directly on the section, and then incubated at room temperature for 10 min.

4. Apply working strength TdT enzyme

Tissue slide was gently tap off excess liquid around the section, then 55 ul/ 5cm^2 of working strength TdT enzyme was applied directly on the section, then incubated at 37 ℃ for one hour.

5. Apply stop/ wash buffer

After that, tissue slide was put into the coplin jar containing with working strength stop/wash buffer, and agitated for 15 sec, followed by incubating at room temperature for 10 min.

6. Apply working strength anti-degoxigenin conjugate

The tissue slide was washed in 3 changes of PBS for one min in each wash, gently tap off excess liquid on tissue. After that, 65 ul/5 cm^2 of anti-degoxigenin conjugate was applied on to the tissue. Tissue was then incubated in light protection

humidified chamber at room temperature for 30 min. After that, tissue was washed in 4 changes of PBS in a coplin jar for 2 min in each wash.

7. Counterstain and mount after fluorescein staining

After conjugate staining, 15 ul/ 22 × 50 mm of 0.5-1 ug/ml DAPI was applied on to tissue, then tissue was covered by cover slip. Tissue was observed under fluorecein microscopy using wavelengths at 520 nm.

CHAPTER IV

RESULTS

Part I. Establishment of tumor mice model

1.1 Different amount of HeLa cells for establishing tumor mass

In order to optimize the amount of the HeLa cells that need to establish a tumor mass, various amount of HeLa cells (2.5×10^5, 5×10^5 and 1×10^6) was inoculated into the dorsal skin-fold window chamber. The control group mice received MEM medium. Each group contained 5 mice. After inoculation, 3 mice died due to traumatizing lesion within 24 h (Table 3). One was from group receiving HeLa 2.5×10^5 cells and another 2 mice were from group implanted with HeLa 5×10^5 cells. The rest living mice were regularly weighed every week until 8 weeks. It was observed that HeLa cells at dose of 2.5×10^5 cells could generate tumor mass at 3 weeks after the inoculation, while it was only 2 weeks when the amount of HeLa cells were 5×10^5 and 1×10^6 cells (Figure 13 and 14). Since the tumor size was enlarged continuously, the chamber was removed when the tumor mass became larger usually at 3 weeks. At highest dose of (1×10^6 cells) it was 100% successfully established tumor mass. The linear regression analysis indicated that means of tumor size from each group significantly increased in relation to number of HeLa cells used as shown in figure. 15 (R^2 = 0.98, y = 0.1171x+4.35). Figure 16 indicated that the rate of tumor growth increased in linear relation to the amount of HeLa cells used. In particular, it increased up to 2 times rate of tumor growth when the high number of inoculated cells was used, 1×10^6 cells.

Table 3. Tumor size and weight of inoculated mice

No. HeLa cells No of mice with tumor (%)		Week (s) after implantation [Values are mean± SD]								
		0	1	2	3	4	5	6	7	8
2.5×10^5 4 /5 (80)	Size (mm)	0	0	0	4.6 ± 1.70	8.0 ± 2.30	8.75 ± 1.5	9.25 ± 1.5	9.25 ± 1.5	10.25 ± 2.87
	Body weight (g)	23.24 ± 1.61	24.06 ± 0.64	24.26 ± 0.42	25.32 ± 1.23	26.16 ±1.31	25.84 + 1.24	25.82 + 1.02	25.72 + 1.07	26.36 + 1.01
5×10^5 2 /3 (66)	Size (mm)	0	0	4.00 ± 0.00	5.00 + 1.00	5.3 + 0.57	6.6 + 0.57	7.3 + 1.15	8.3 + 1.52	9.6 + 0.57
	Body weight (g)	18.6 ± 0.66	20.66 ± 1.15	21.60 ± 1.15	21.76 + 1.07	21.83 + 1.15	21.60 + 0.26	22.03 + 0.65	23.00 + 0.72	23.73 + 0.70
1×10^6 4/4 (100)	Size (mm)	0	0	3.0 ± 0.81	5.5 + 1.00	6.5 + 3.10	12.0 + 6.53	14.25 + 7.13	14.75 + 7.36	14.75 + 7.36
	Body weight (g)	17.3 ± 1.04	19.67 ± 0.88	20.60 ± 0.98	21.00 + 1.37	21.85 + 1.36	22.32 + 1.14	22.37 + 1.14	23.03 + 1.31	24.02 + 1.49

Figure 13. Mouse injected with 5×10^5 cells at 2 weeks

Figure 14. Mouse injected with 1×10^6 cells at 2 weeks

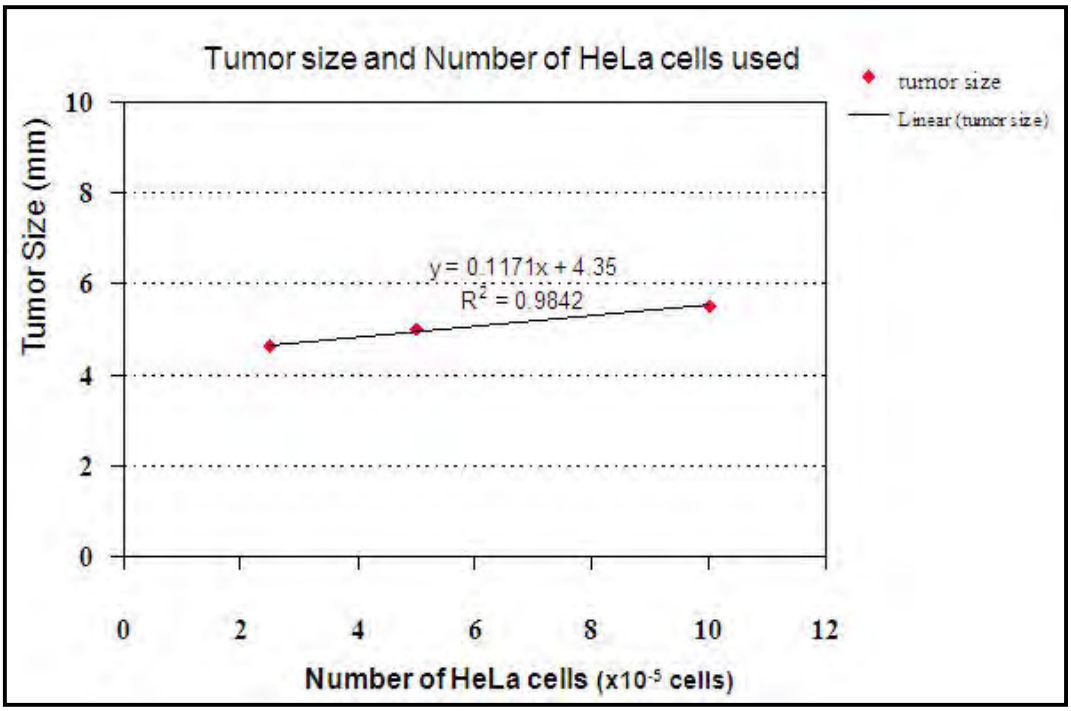


Figure 15. Means of tumor size plotted against number of HeLa cells used

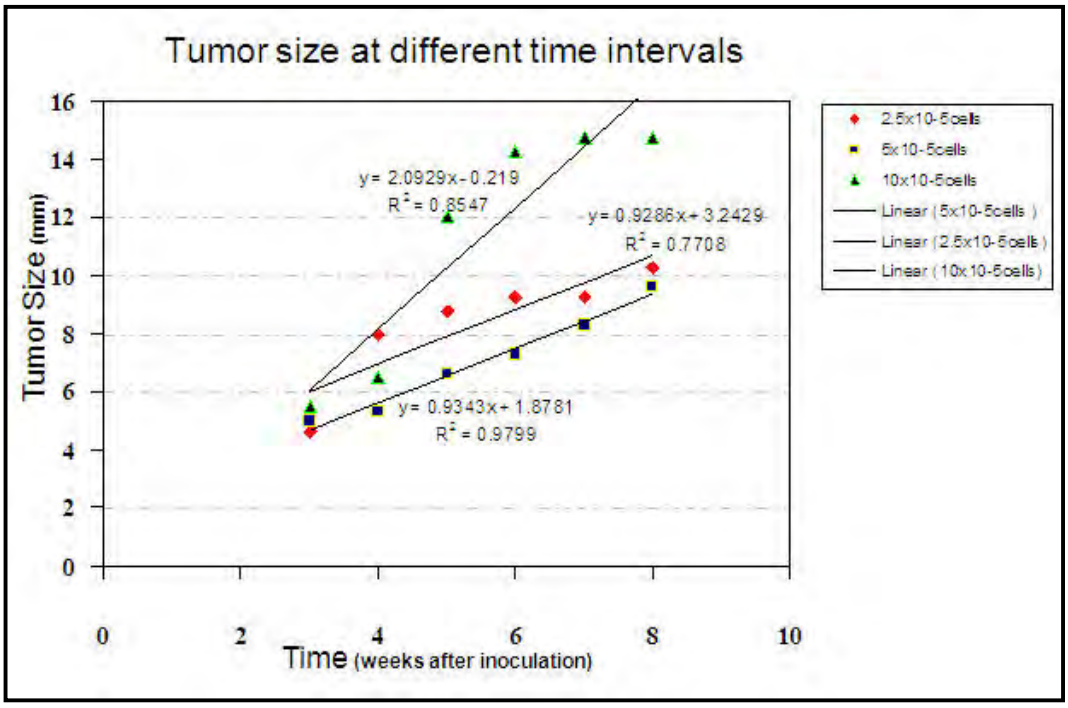


Figure 16. Means of tumor size plotted against time intervals after the inoculations using different number of HeLa cells.

1.2 Histopathological changes and tumor angiogenesis

For studying histopathological changes and tumor angiogenesis**,** a new group of mice was inoculated with 1×10^6 HeLa cells. After 2 weeks of inoculation, 2 out of 5 mice from both control and HeLa-groups were sacrificed and observed for histopathological changes and tumor angiogenesis. From H&E examination, the characteristic of squamous cell carcinoma was found in 2 weeks HeLa mice whereas none was present in control mice (Figure 17 a, b and c). In addition, the appearance of developed tumor on dorsal skin of those mice also showed necrosis and hematoma lesions. Moreover, by using intravital fluorescent videomicroscopy, the results of tumor micro-circulation revealed the increase in neocapillary density in 2 week HeLa group as compared to control group (Figure 18). One mouse was sacrificed at 3 months for histopathological study which also showed the same characteristic of squamous cell carcinoma. The other 2 mice formed grossly identifiable tumors were able to live up to 6 months.

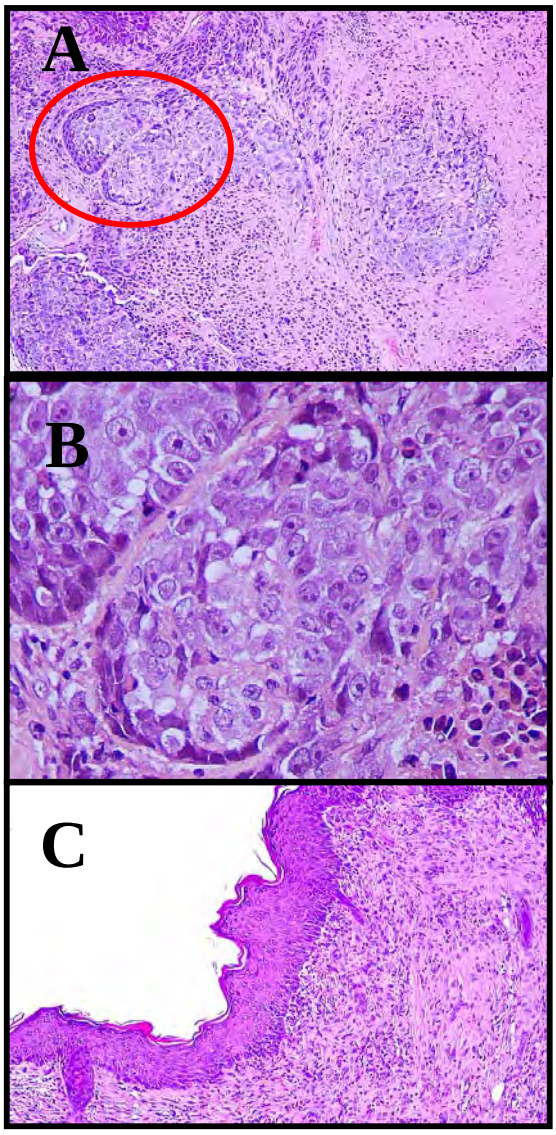


Figure 17. Tissue section of tumor from 2 weeks implanted (1×10^6 cells) and control mouse.

A. Tissue section from implanted mouse showed histopathology of squamous cell carcinoma (×100)

B. Tissue section from implanted mouse showed histopathology of squamous cell carcinoma (×400)

C. Tissue section from control mouse showed no characteristic of squamous cell carcinoma. (×100)

D.

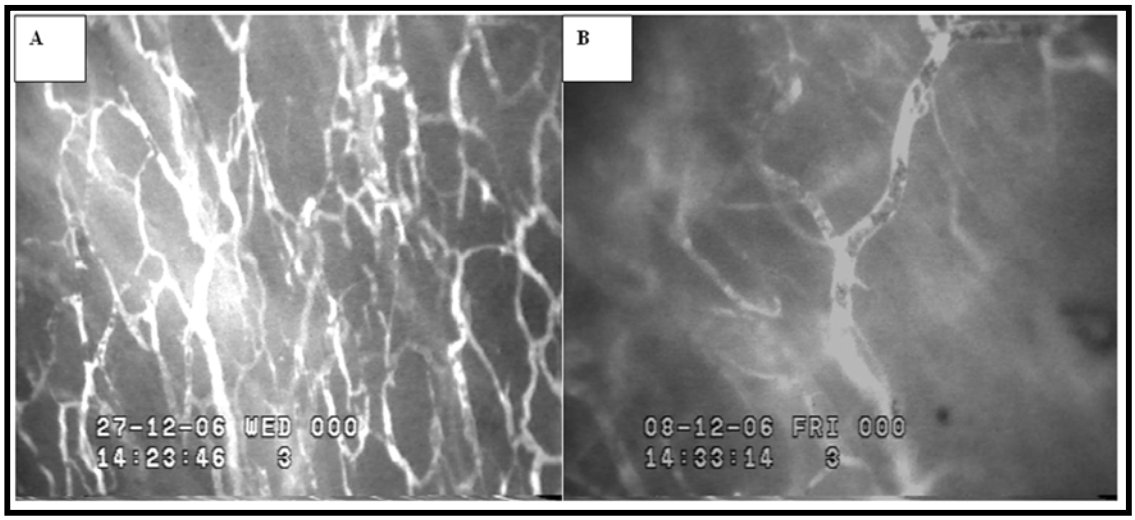


Figure 18. Intravital Fluorescence Videomicroscopy of 2 week-1×0^6 HeLa implanted mouse (A) compared with control mouse (B) ×100.

HPV detection

In order to confirm that tumor growth came from HeLa cells, DNA extraction from tumor tissue was amplified with HPV-L1 specific primers. Moreover, tissue sections were determined by *in situ* hybridization with probe specific to both HPV-16 and 18. The dark brown staining cells were HPV DNA positive cells (Figure 19C). Finally, HPV genotype was done by using HPV genotyping kit (Inno-Lipa HPV Genotyping V2; Innogentics; Germany). All assays indicated that HPV-DNA of type 18 was found in the extracted DNA and located in the tumor cells (Figure 19).

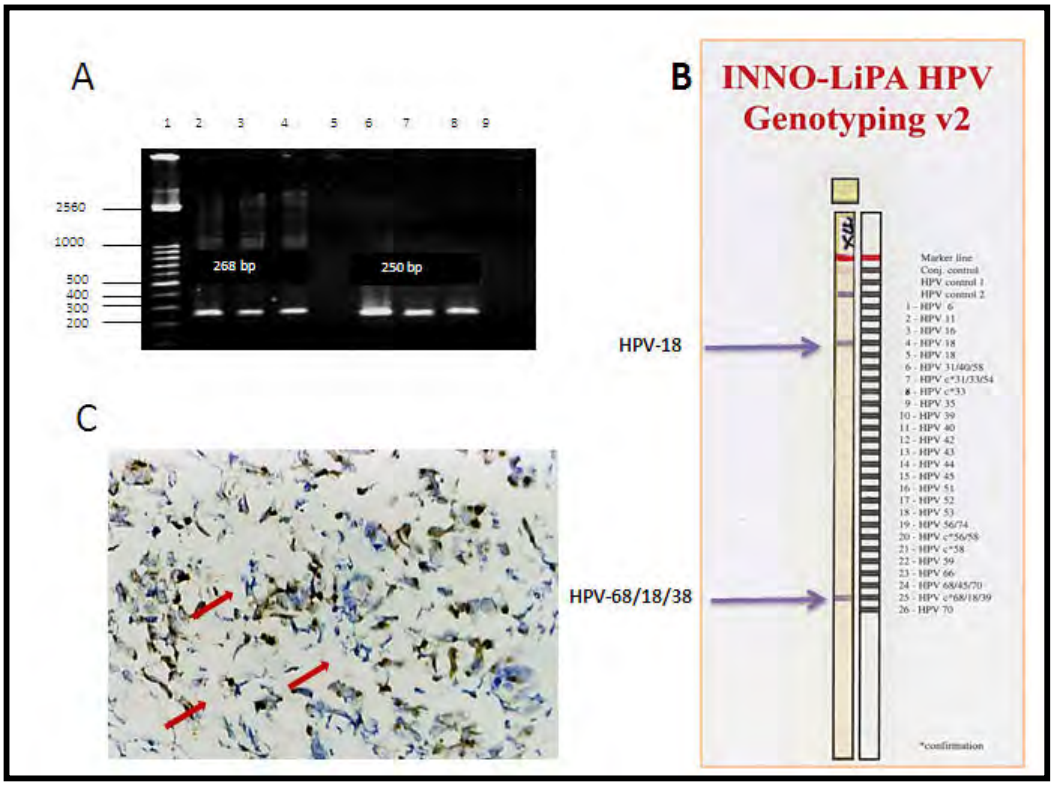


Figure. 19. HPV detection in tumor tissues by (a) PCR, Lane 1: DNA marker (bp); Lane 2 and 6: HeLa DNA; Lane 3, 4, 7 and 8: Tumor extracted DNA; Lane 5 and 9: normal mouse tissue; Different set of primers was done; Lane 2, 3, 4, 5: human beta-globin primers, Lane 6, 7, 8, 9: HPV-L1 primers. (b) HPV genotyping (c) *In situ* hybridization (arrow indicated positive HPV-DNA inside the cells).

Part II. Anti-tumor effects of human γ/δ T cells: *In vitro* assay

Pamidronate induce expansion of γ/δ T-cells from donor PBMC

In order to observe the individual differences in the γ/δ T cell stimulating ability of pamidronate, PBMC from 3 blood donors were cultured for 14 days with various concentrations of pamidronate (0.625-200 μM/ml) and exogenous IL-2 (100 μ/ml). The results indicated that pamidronate was able to induce expansion of γ/δ T cells from all blood donors. The optimum dose that could be stimulate the highest number of γ/δ T cells was 10 uM/ml, which generated 77 % of γ/δ T cells. While, a doses above 10 uM/ml (100 and 200 uM/ml) were toxic dose for PBMC. In control group (activated with RPMI 1640 and 100 units of IL-2) only 27% of γ/δ T cells could be observed (Figure 20-21).

Purification of γ/δ T-cells

After 14 days cultivation of isolated PBMC, the γ/δ T cells were purified by using magnetic cell sorting (MACS) as described in materials and methods. Purified γ/δ T cells were analyzed by fluorescence microscope and proved by flowcytrometry (Figure 22). In this study at least 90 % of γ/δ could be purified by this MACS.

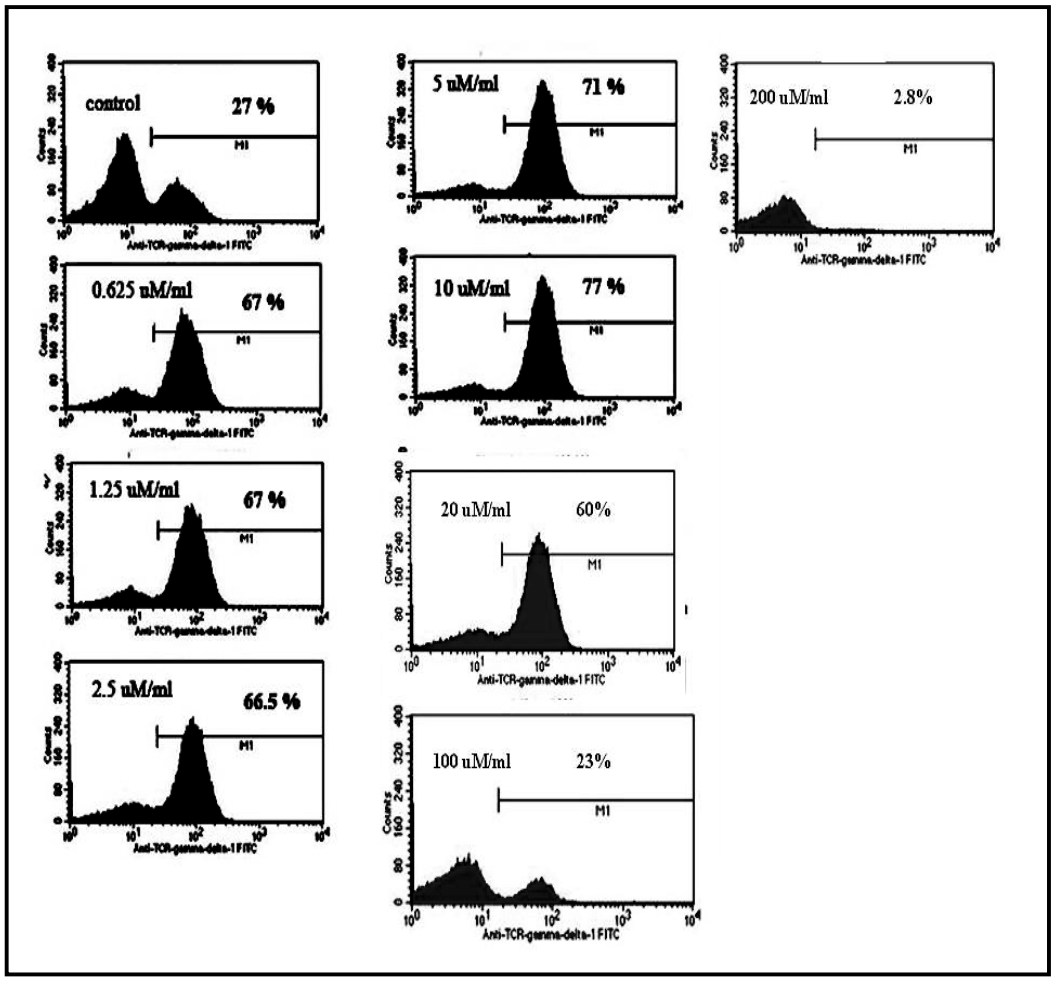


Figure 20. Flowcytometry analysis of PBMC after 14 days cultured in the present of pamidronate at various concentrations (0.625-200 uM/ml), using FITC anti pan γ/δ monoclonal antibody.

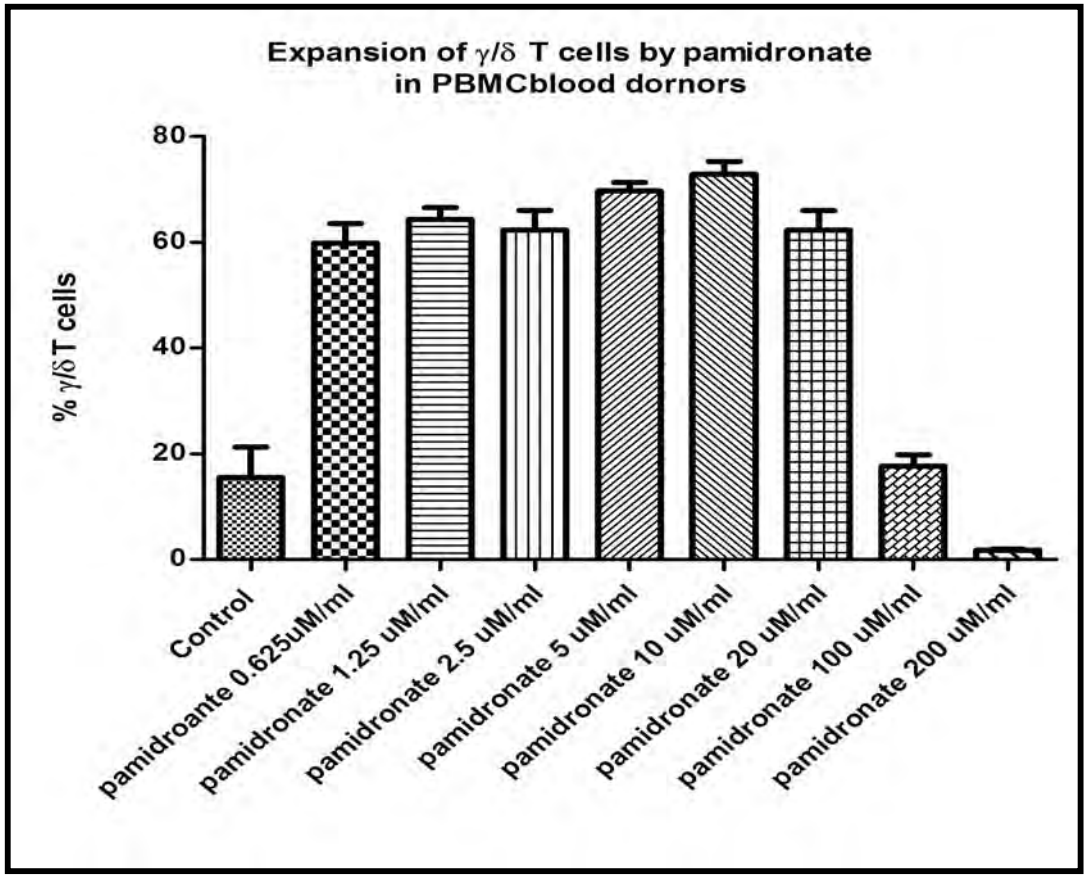


Figure 21. Expansion of γ/δ T cells from primary PBMC culture of 3 different donors by pamidronate at various concentration (0.625-200 uM/ml) at 14 days, as determined by flowcytometry analysis. Graphs are expressed as mean $\pm$ SD of average from duplication of 3 representative experiments.

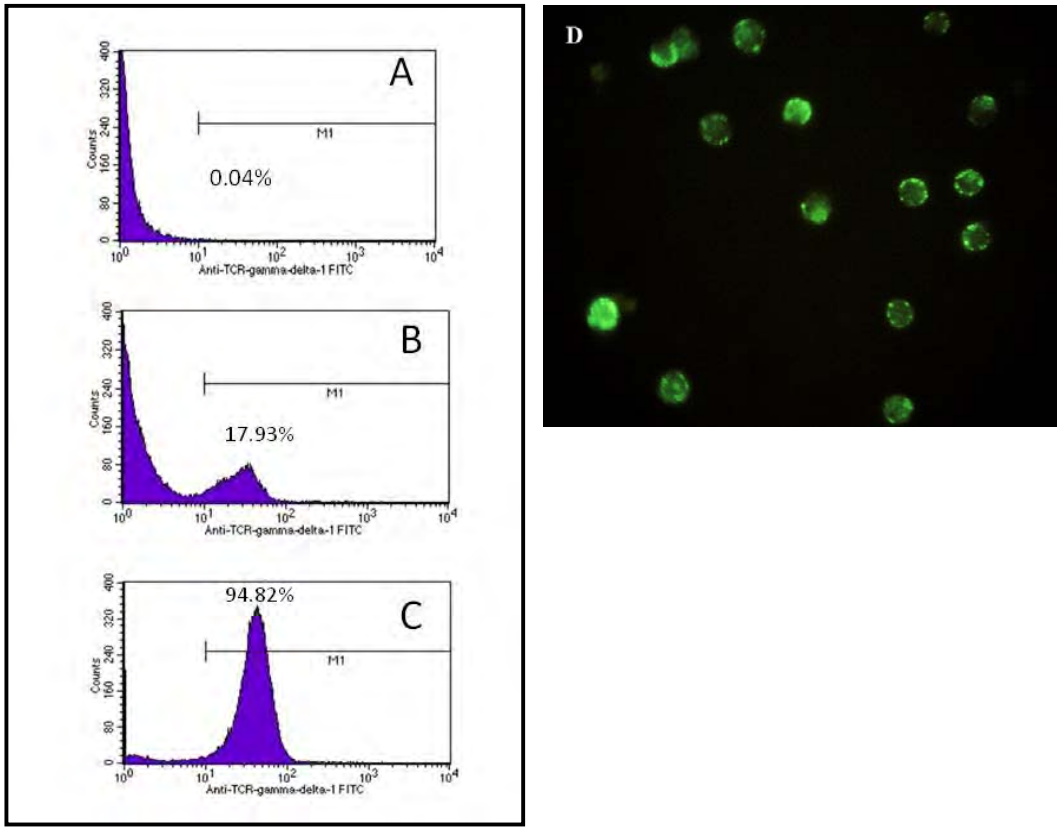


Figure 22. Purified γ/δ T cells after 14 day cultivation. A) non-stained pamidronate treated PBMC. B).PBMC stained with FITC anti γ/δ TCR antibody. C) purified γ/δ stained with FITC anti γ/δ TCR antibody.

Expansion of Vγ9Vδ2 T cells by pamidronate in primary PBMC culture

In order to investigate that the pamidronate can activate expansion of Vγ9/ Vδ2 subpopulation in individual PBMC, PBMC from blood donor were cultured with pamidronate 10 uM/ml and supplement with IL-2 100 u/ml every 3 days until 14 days. PBMC were separately stained with FITC anti pan γ/δ antibody, PE anti Vγ9 specific antibody and FITC anti Vδ2 specific antibody. These results showed that approximately 87 % of pamidronate stimulated γ/δ T cells were Vγ9/ Vδ2 subpopulation. (Figure 23)

Pamidronate did not effect the growth of cervical cancer cell lines

Since, this study requires pamidronate to activate IPP accumulation with in cervical cancer cell lines, hence the optimal concentration of pamidronate had to evaluate prior for starting the experiments. All 3 cervical cancer cell lines (HeLa, SiHa, and CaSki) with 10,000 cells /well were cultured in 48 wells plate. The cells were treated with single dose of various concentration of pamidronate (0,1,10,20,40,80,100,200 uM/ml). After 5 days, monolayer cells were washed, fixed on plate and stained with 0.5% crystal violet according to the materials and methods. After air dried, the incorporated crystal violet was solubilized in 1 ml of 0.1 M sodium citrate in 50% ethaonol and 100 ul aliquots were transferred to the 96 well ELISA plate. The number of cells was determined by the optical density (OD) at 550 nm. The results indicated that optimized concentration for activating all 3 cervical cancer cell lines was 10 uM/ml, whereas concentrations above that were toxic to all tested cervical cancer cell lines if increase the concentratin to 20 uM/ml (Table 4 and Figure 24)

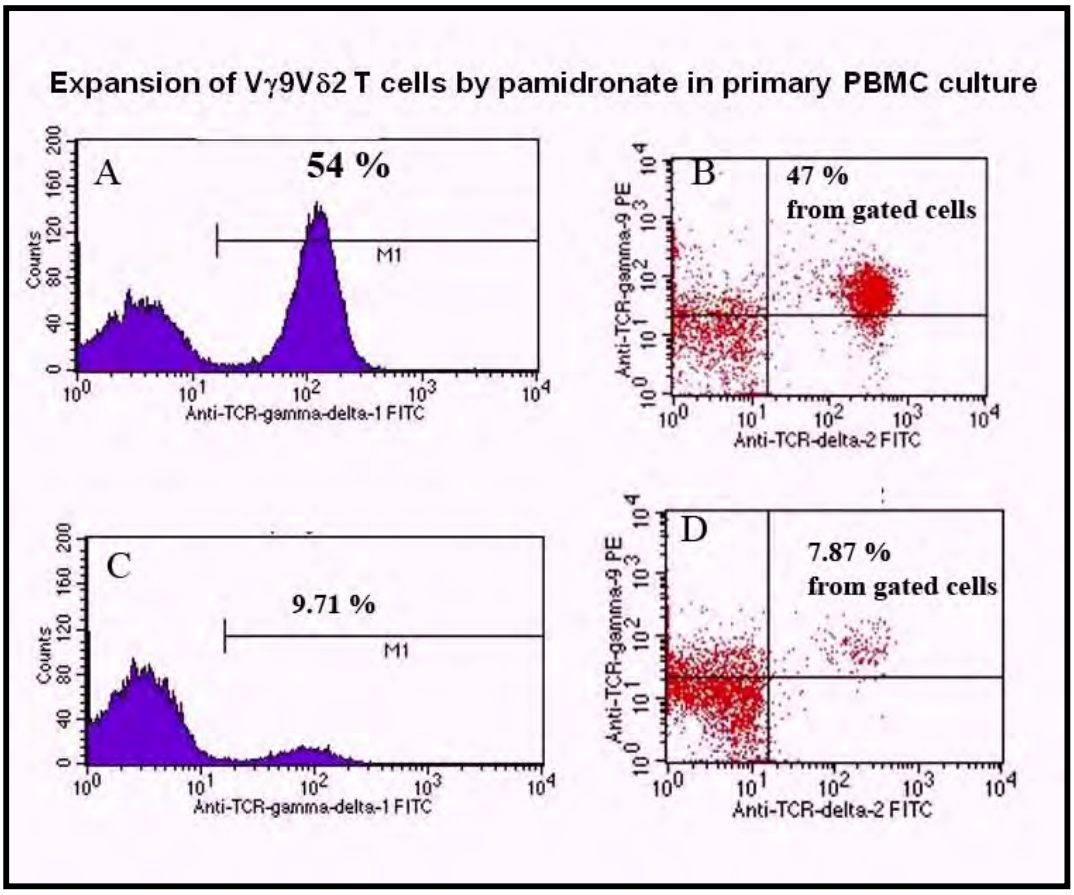


Figure 23. Analysis of γ/δ subpopulation that was stimulated by pamidronate, using specific monoclonal to Vγ9/ Vδ2 TCR, indicated that most of them were Vγ9/Vδ2 T cell.

A) Stimulated PBMC stained with FITC pan anti g/d TCR antibody.

B) γ/δ T cells from stimulated PBMC stained with PE anti Vγ9 specific antibody and FITC anti Vδ2specific antibody

C) Control PBMC stained with FITC pan anti g/d TCR antibody.

D) γ/δ T cells from control PBMC stained with PE anti Vγ9 specific antibody and FITC anti Vδ2specific antibody

Effects of pamidronate on proliferation of 3 cervical cancer cell lines

Pamidronate uM/ml	HeLa		SiHa		CaSki	
	Mean	±SD	Mean	±SD	Mean	±SD
0	2298	48.18022	2130	114.6138	2807	144.3757
1	2242	93.78699	2112	134.9778	2702	74.96889
10	1979	73.58215	2062	74.59446	2469	143.1445
20	941	59.73274	311	12.01388	832	141.3164
40	808	44.88132	205	26.51415	940	54.04936
80	183	24.68468	174	17.24336	593	105.5099
100	191	2.309401	154	13.52775	656	52.84253
200	195	3.785939	175.	10.2632	657	10.69268

Table 4. Effects of pamidronate on proliferation of 3 cervical cancer cell lines. Cells were treated up to 5 days with various concentrations of pamidronate (0,1,10,20,40,80,100,200 uM/ml), without renewing the medium. Cells were plated at a density of 10,000 per well in 48-well plates. On selected days cell monolayers were stained with crystal violet dye. The concentration of incorporated crystal violet was determined by measuring the OD at 550 nm. Table are expressed as mean and ± SD from triplicates experiment (n=3).

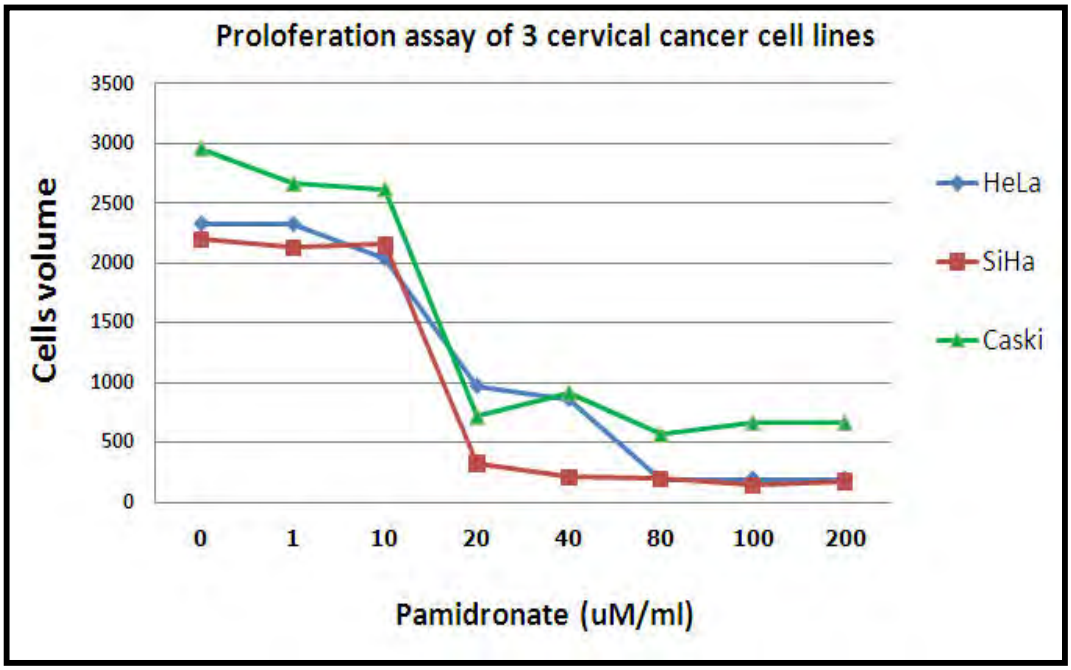


Figure 24. Effects of pamidronate on proliferation of 3 cervical cancer cell lines. Cells were treated up to 5 days with various concentrations of pamidronate (0,1,10,20,40,80,100,200 uM/ml), without renewing the medium. Cells were plated at a density of 10,000 per well in 48-well plates. On selected days cell monolayers were stained with crystal violet dye and absorbance at 550 nm. Graphs are expressed as mean from triplicates experiment.

Lysis of cervical cancer cell lines by pamidronate stimulated Vγ9/Vδ2 T cells.

Three HPV-positive cervical cancer cell lines (Hela; HPV-18, SiHa; HPV-16 and CaSki; HPV16) were used as the target of γ/δ T-cells and NTY, normal human fibroblast isolated from cervical cancer patient, as negative control. PHA activated autologous PBMC were used as internal control in cytotoxic test system. All cell lines were incubated for 12 h in 96 well plates with R-10 medium (see Appendix B) with or without 10 uM/ml of pamidronate. On the next day, after washing with PBS, purified γ/δ T-cells from healthy blood donor were added in to the target cells at the ratio of 20:1 and 40:1 (effecter : target) and fresh R-10 medium supplementd with IL-2 (20 u/ml) were added. After 5 h, supernatant was collected for LDH Cytotoxicity detection and INF-γ and TNF-α determination. The results of cytotoxic activity were presented in table 5 and figure 25 indicating that γ/δ T-cells had ability to kill all cervical cancer cell lines including to normal fibroblast cell but not PHA blast autologous PBMC. The cytotoxicity to target cells increased when those cells were treated with pamidronate. The sensitivity of cells to Vγ9/Vδ2 T cells was HeLa> SiHa> CaSki> NTY > PHA blast PBMC. Statistic analysis indicated that the cytotoxicity of Vγ9/Vδ2 T cells to HeLa treated with pamidronate at the ratio of effector: target 1:20 had a significantly greater than non treated HeLa cell ($p<0.01$), whereas the ratio 1:40 was not significant. Similar observation was found in SiHa and CaSki cells (Tabe 5).

% Cytotoxicity (Mean ± SD)

Cells	E:T = 1:20	*P* value	E:T = 1:40	*P* value
HeLa: Treated	40.702 ± 10.80601	0.0041	51.52 ± 16.56887	NS
HeLa: Non-treat	20.386 ± 11.31398		34.8575 ± 13.52673	
SiHa: Treated	31.31 ± 15.4958	NS	49.316 ± 16.80733	0.0038
SiHa: Non-treat	20.814 ± 10.71395		31.462 ± 11.12895	
CaSki: Treated	25.61 ± 11.16863	NS	48.46667 ± 13.57399	0.0461
CaSki: Non-treat	13.93333 ± 1.601041		28.16667 ± 7.518201	
NTY: Treated	17.126 ± 6.006969	0.0395	36.576 ± 14.54249	0.0176
NTY: Non-treat	10.338 ± 3.807581		16.598 ± 5.448488	
PHA blast	3.95 ± 0.494975		Nd Nd	

NS= nonsignificant, Nd = Not done

Table 5. Cytotoxicity of Vγ9/Vδ2 T cells to pamidronate treated and none treated of 3 cervical cancer cell lines, normal fibroblast and PHA balst autologous PBMC at the ratio of (E) : (T) 1:20 and 1:40. Mean ± SD from duplicated of 5 donors (except in PHA blast PBMC was done in twice) were shown.

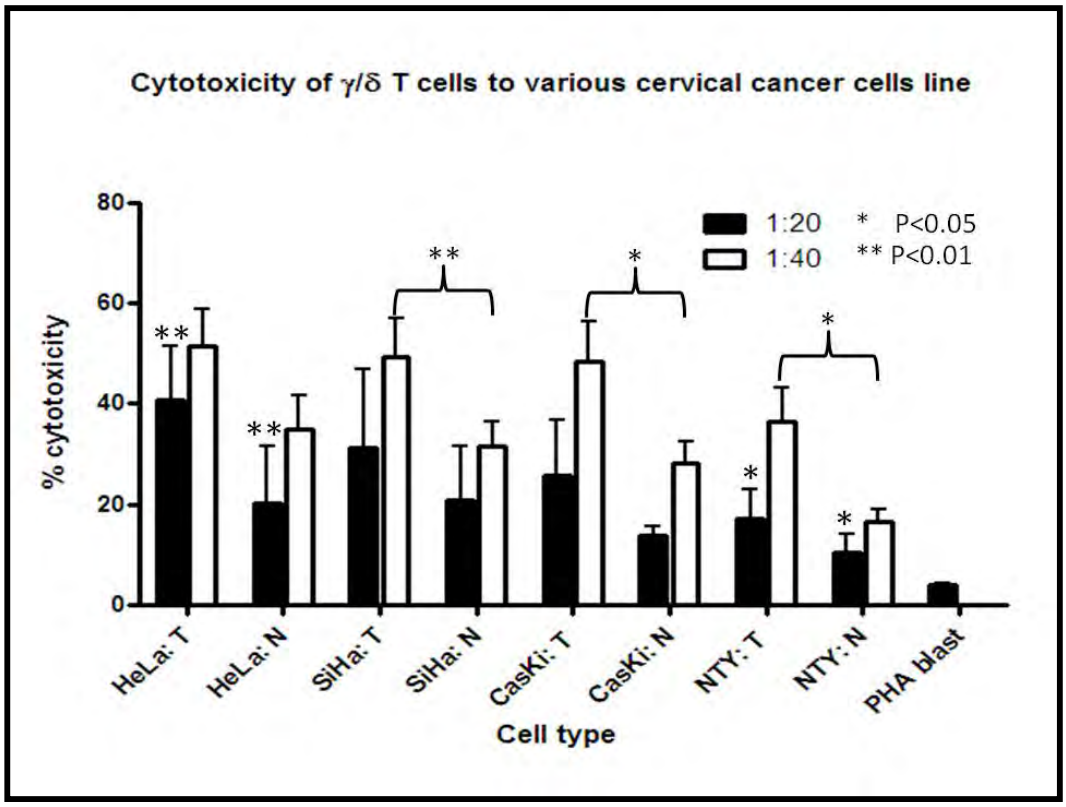


Figure 25. Cytotoxic activity of Vγ9/Vδ2 T cells against cervical cancer cell lines, normal fibroblast and PHA blast autologous PBMC. Cytotoxicity was measured by LDH assay. Graphs are expressed as mean ± SD. (except in PHA blast PBMC was done from 2 donors). The black bar represent 1:20 (effector : target ratio), The blank bar represent 1:40 (effector : target ratio), T= treat with pamidronate, N= non treat with pamidronate. For statistic analysis, the pair *t*-test was performed, * statistic significant with $p<0.01$, ** statistic significant with $p<0.05$

Cytokine production of Vγ9/Vδ2 T cells

Vγ9/Vδ2 T cells were cultured with cervical cancer cell lines in the present of IL-2 (20 units /ml) for 5 hr. After that the supernatant was aliquoted and storeed at -80℃ for until measurement of the cytokine. IFN-γ and TNF-α were selected for analysis, due to the present of IFN-γ and TNF-α indicated the functions activity of the Vγ9/Vδ2 T cells. All samples were performed in duplicates using ELISA technique (materials and methods). The results indicated that pamidronate treated and non treated cervical cancer cell lines and NTY cells were able to induce IFN-γ and TNF-α secreting from Vγ9/Vδ2 T cells. Pamidronate treated HeLa was the most efficient cell that can induced secreting of both IFN-γ and TNF-α whereas, NTY cell was the less. In addition, there were a few of IFN-γ and TNF-α secreted from PHA blast autologous PBMC activated Vγ9/ Vδ2 T cells (Table 6-7 and Figure 26-27).

IFN-γ production (pg/ml)

Cell types	E:T ratio 1:20 (mean ± SD)		E:T ratio 1:40 (mean ± SD)	
HeLa: T	8572.8	± 4663.625	12164	± 5861.673
HeLa: N	3101	± 3178.92	5634.667	± 4472.002
SiHa: T	3817.6	± 2458.767	7404.6	± 4423.056
SiHa: N	2121.2	± 2532.331	4142	± 4075.137
CaSki: T	3265.8	± 3073.083	4921.6	± 4349.211
CaSki: N	1899.8	± 2357.344	4126.4	± 4297.646
NTY: T	4726	± 3339.206	7286.8	± 5327.512
NTY: N	1736	± 2385.226	2276.2	± 2656.073
PHA blast	606.5	± 50.5		

Table 6. IFN-γ secretion from activated Vγ9/Vδ2 T cells after co-cultured with various types of cell lines and PHA blast autologous cells. The results were from 5 independent experiments.

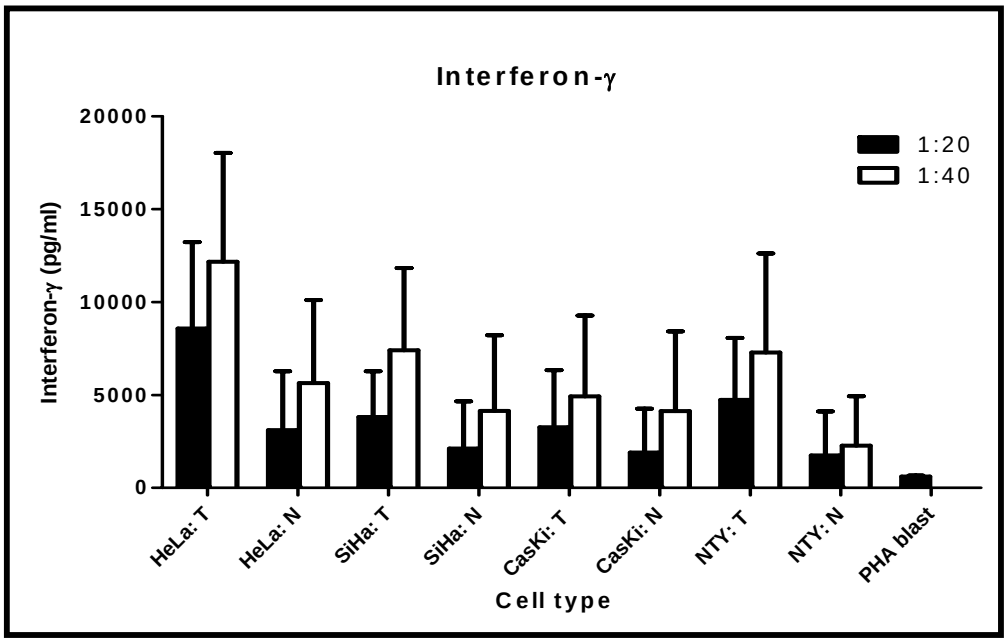


Figure 26. IFN-γ secretion from activated Vγ9/Vδ2 T cells after co-cultured with various types of cell lines and PHA blast autologous cells. The graph were from 5 independent experiments (mean ; pg/ml, ± SD).

TNF-a production (pg/ml)

Cell types	E:T ratio 1:20 (mean ± SD)		E:T ratio 1:40 (mean ± SD)	
HeLa: T	4297.8	± 3608.912	6338.2	± 2897.943
HeLa: N	-32.2	± 566.5438	561.8	± 545.6915
SiHa: T	850.6	± 910.0636	2820.6	± 1801.193
SiHa: N	-383.4	± 252.1447	754.6	± 397.1027
CaSki: T	2456.333	± 819.9554	3633	± 538.1378
CaSki: N	-67	± 290.5323	833	± 690.8924
NTY: T	1250.6	± 776.0623	1808.2	± 1026.997
NTY: N	47.8	± 537.0101	1197.8	± 1127.683
PHA blast	-774.5	± 212.5		

Table 7. TNF-α secretion from activated Vγ9/Vδ2 T cells after co-culture with various types of cell line and PHA blast autologous cells. The results were from 5 independent experiments.

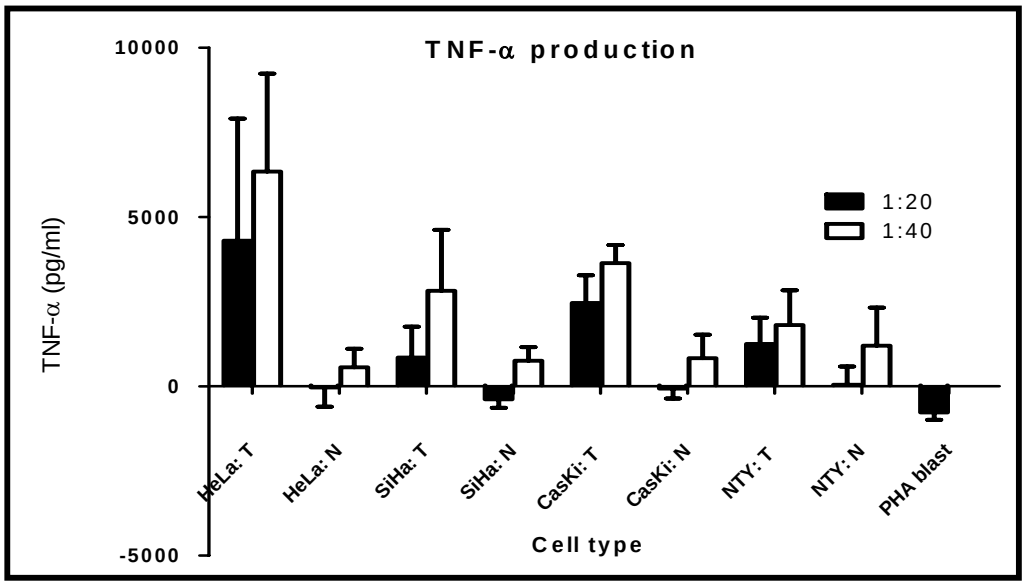


Figure 27. TNF-α secretion from activated Vγ9/Vδ2 T cells after co-culture with various types of cell line and PHA blast autologous cells. The results were from 5 independent experiments (mean; pg/ml, ± SD).

Evaluation of the CD 107 cytotoxicity assay

In order to confirm the phenotypic and functional characterization of cytotoxicity of responding Vγ9/Vδ2 T cells to various target cells, transient expression of CD107 molecule on Vγ9/Vδ2 T cells surface was evaluated by flow cytometry, 3 cervical cancer cell lines (HeLa, SiHa and CaSki) were co-cultured with purified Vγ9/Vδ2 T cells and FITC anti CD107a MAB in 96 well plate. After one hour of incubation, 1 ul of Brefedin A was added to each well and the plate were returned to incubation for additional 4 hr as described previously in materials and methods. The results showed that all pamidronate treated cervical cancer cell lines were able to induce degranulation of Vγ9/Vδ2 T cells whereas, non treated cervical cancer cell lines were unable to induce degranulation. However, not all Vγ9/Vδ2 T cells response to pamidronate treated cervical cancer cell lines. The maximum percentage of Vγ9/Vδ2 T cells that responded to pamidronate treated HeLa cell was 10.96 %, whereas only 1.07 % was found in non-treated HeLa cell. Ina addition, the maximum percentage of Vγ9/Vδ2 T cells that responded to pamidronate treated SiHa and CaSki cells were 13.95% and 6.91%, respectively. While only 0.91% and 0.89 % of Vγ9/Vδ2 T cells were found in non treated SiHa and CaSki cell lines (Figure 28).

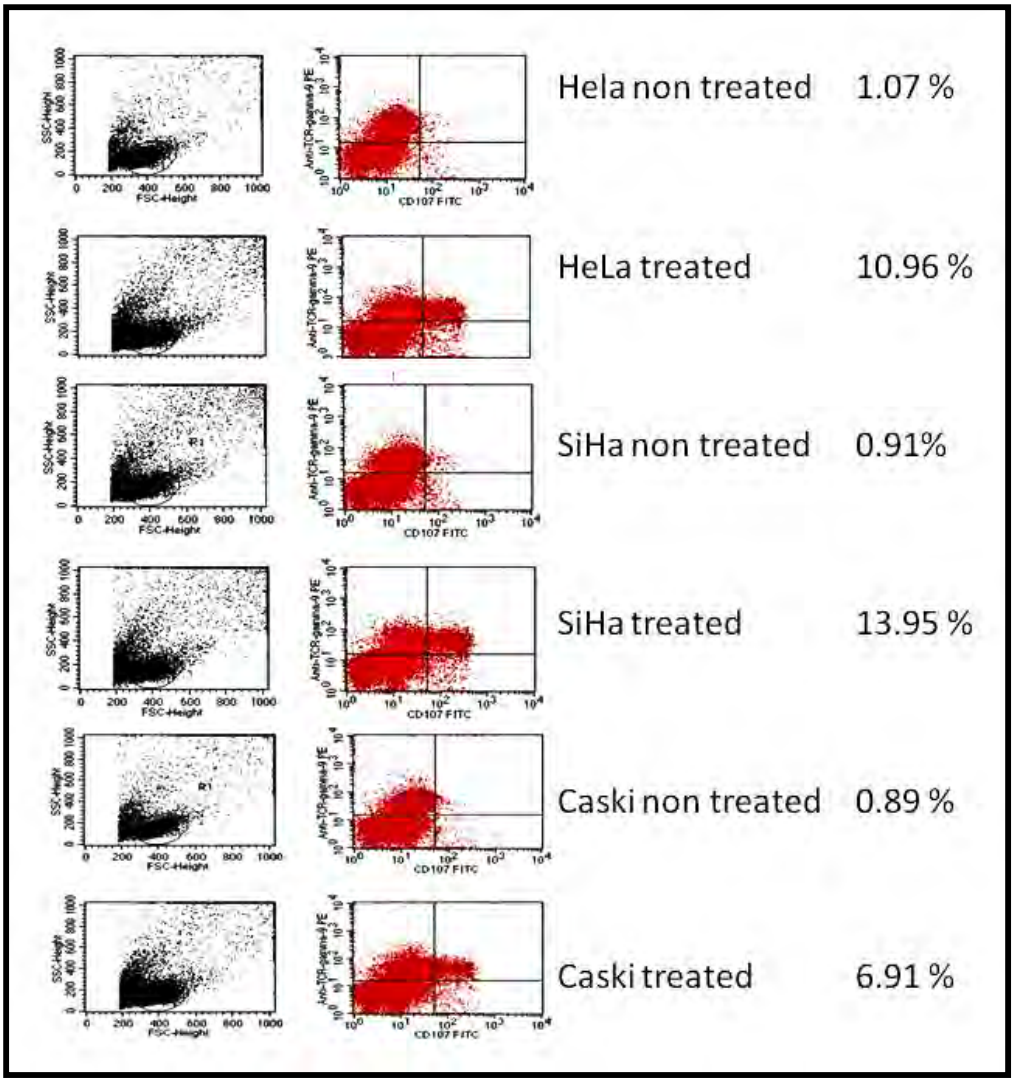


Figure 28. Evaluation of the CD 107 cytotoxicity assay. Purified Vγ9/Vδ2 T cells were co-cultured with cervical cancer cell lines, and observed for the expression of CD107 on the cell surface by staining with FITC anti CD107a antibody and PE anti Vγ9 TCR antibody.

Part III. Anti-tumor effects of human γ/δ T cells in a mouse bearing tumor

Cytotoxic effects of Vγ9/Vδ2 T cells in mouse model

As showed previously in the part I study, we have been able to successfully establish a new mouse model for cervical cancer study. Then we future question wheather this Vγ9/Vδ2 T cells are able to kill the tumor cells in mouse.

Purified Vγ9/Vδ2 T cells and the flow through PBMC from γ/δ purification process were stained with Green; 5-fhloromethylfluorecein diacetate (CMFDA), a cell tracker dye probes for long-term tracing of living cells before injected in to tumor mice. Approximately 2 weeks of tumor developed mice were used (n=4), hence mice were aged between 6-10 weeks. Mice were anesthetized with pentobarbital sodium (40 mg/kg; IP). After anesthetization, approximately 3×10^6 of stained Vγ9/Vδ2 T cells were injected around tumor site (n=3), the control mouse was received the labeled flow through PBMC. Mice were observed the distribution of cell by *in vivo* imaging system (Kodak, USA) (Figure 29).

Deposit of Vγ9/Vδ2 T cells with in tumor site

After 3 days of injecting Vγ9/Vδ2 T cells, mice were scarified and collected tumor tissue for histopathology study and observed the deposition of Vγ9/Vδ2 T cells with in the tumor site. Approximately 5 u of tumor sections were stained with

anti Human CD3 (materials and methods). The result are showed in figure 30, indicated that after 3 day of injection Vγ9/Vδ2 T cells were still located with in tumor areas.

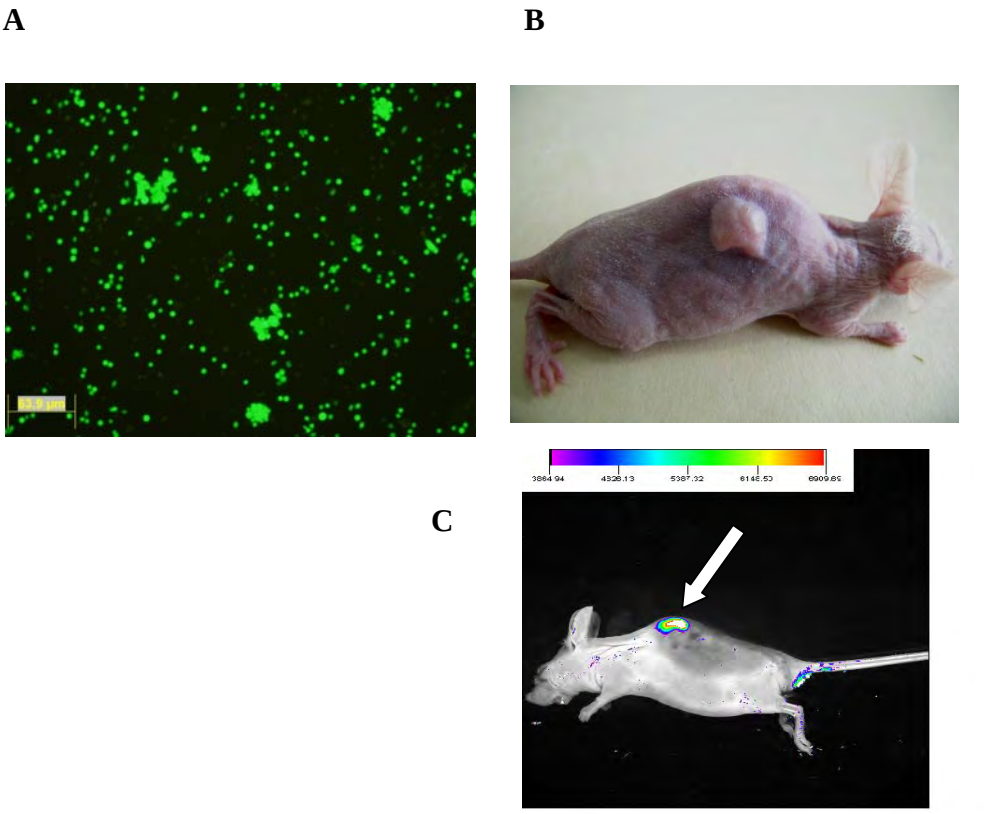


Figure 29. Live views of injected mouse with Vγ9/Vδ2 T cells

A. Vγ9/Vδ2 T cells stained with CMFDA (under fluorecein microscopy ×10)
B. Two weeks of tumor developed mouse before injected with Vγ9/Vδ2 T cells
C. The distribution of injected Vγ9/Vδ2 T cells (under live imaging camera) The rainbow color is the injected Vγ9/Vδ2 T cells (arrow).

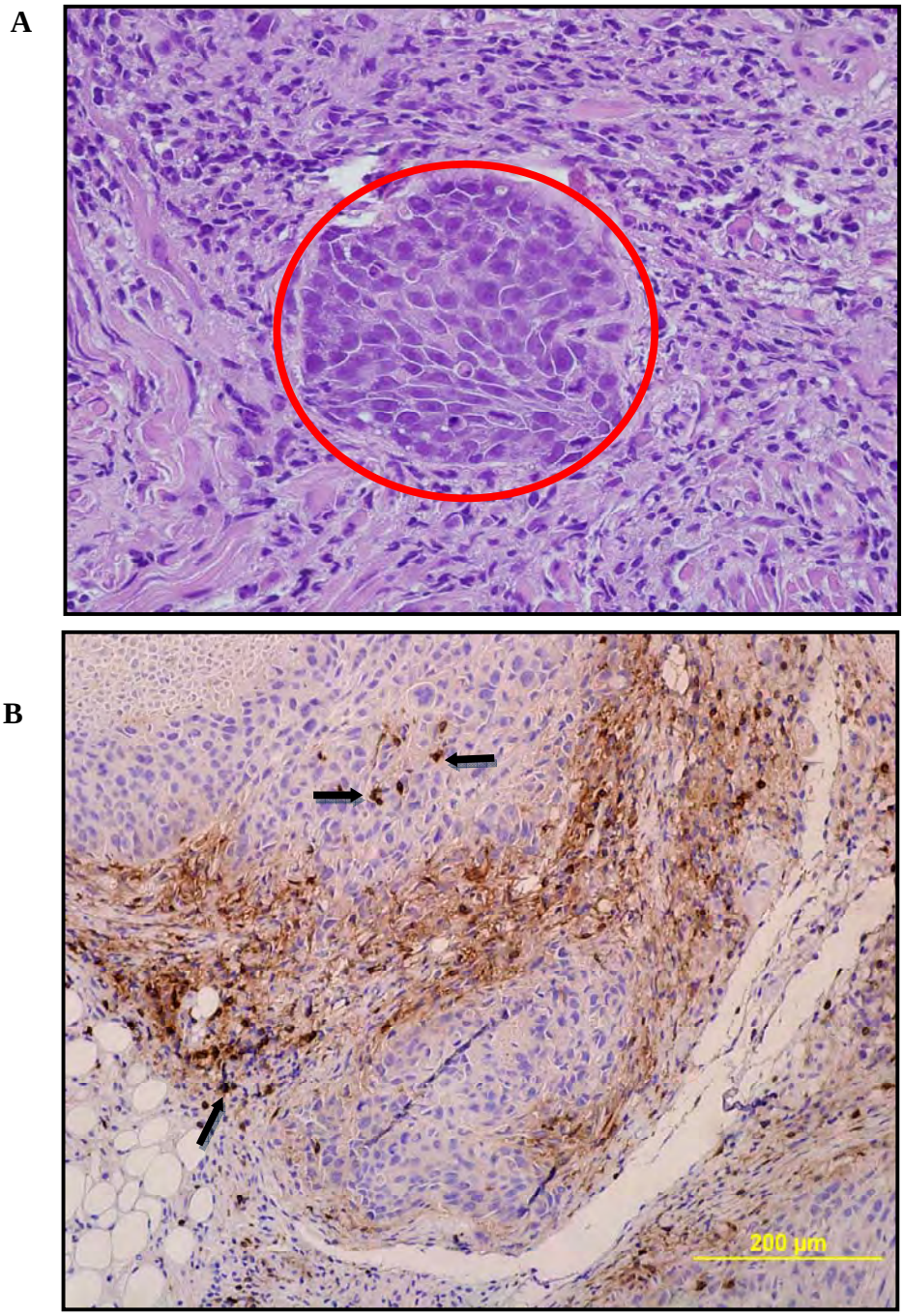


Figure 30. Tumor section, A stained with H&E. The circle area indicated the characteristic of squamous cell carcinoma (×400). B Tumor section stained with anti human CD3 antibody, the arrows indicated the deposit of Vγ9/Vδ2 T cells (×200) .

***In situ* apoptosis detection**

Further investigation on the function of Vγ9/Vδ2 T cells that deposited with in tumors areas was performed by using *In situ* apoptosis detection assay kit (CHEMICON). After 3 days of injecting Vγ9/Vδ2 T cells, mice were scarified and collected tumor tissue for histopathology and apoptosis study. Tissue was immediately fixed in neutral buffer formalin for 24 hr before embedded in paraffin block. Approximately 5 um of the formalin-fixed paraffin-embedded tissue was sliced and fixed onto slide for apoptosis detection. The results showed that the characteristic of DNA fragmentations could be observed within the Vγ9/Vδ2 T cells injected tumor tissue but not in the control tissue. This indicated Vγ9/Vδ2 T cells could be induced apoptosis in the tumor cells (Figure31).

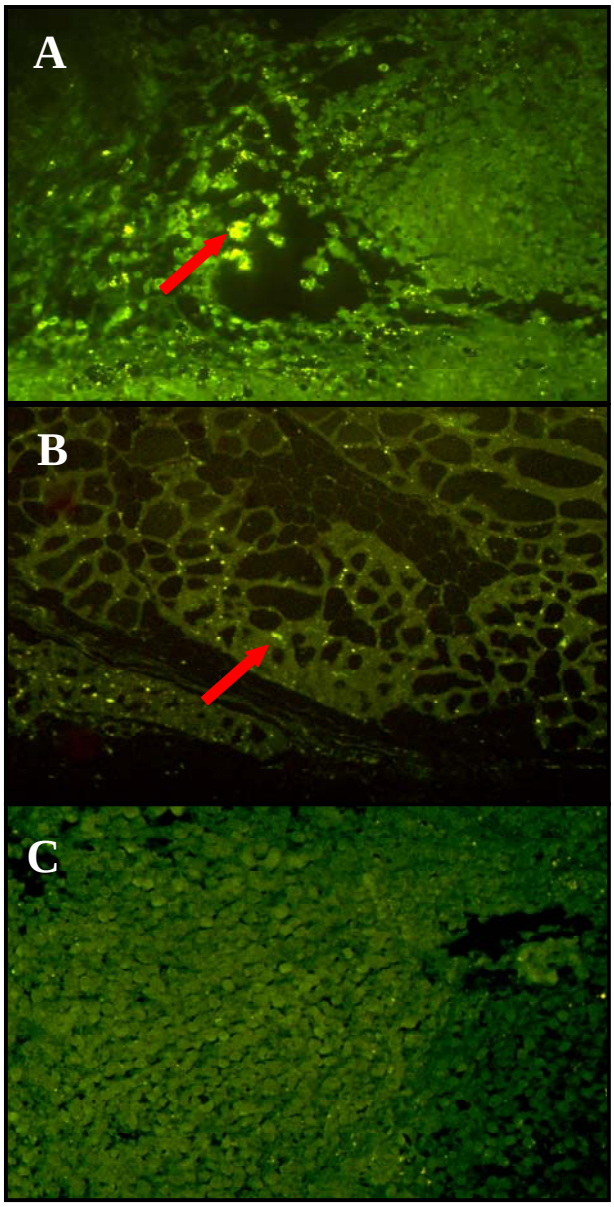


Figure 31 . *In situ* apoptosis assay.The role of Vγ9/Vδ2 T cells to induce apoptosis within tumor sections were analyzed by using *In situ* apoptosis detection kit. Tissue section were stained with a rhodamine fluorochrome and observed under fluorescent microscope. A. Positive sections from mice tumor, arrow indicated the characteristic of apoptotic cells (×200). B. Positive control section from apoptosis kit (×200).
C. Negative section from control mouse (×200).

CHAPTER V

CONCLUSION AND DISCUSSION

Cervical cancer is still a public health problem in women worldwide. Although, many risk factors associated with cervical cancer have been known, but the most important etiology of this cancer is caused by HPV infection [41, 159] HPV, in contrast to many viruses, is species specific and can not propagate in any kinds of animal system. Although HPV propagation has been reported in cell culture system (raft system), the technique needs not only cell proliferation but also cell differentiation. Therefore, development of specific treatment such as chemotherapy, immunotherapy or vaccine trial for HPV infections has been impeded by lack of a model of viral pathogenesis. There have been several attempts to create cervical cancer in animal models, but to date no animal models has similar pathogenesis as natural HPV infection occurs in women.

This study was another attempt to induce cervical cancer in mouse model by directly implanting of human cervical cancer cells line. A HeLa cell, the HPV-18 integrated cervical cancer cell lines was used with the application of dorsal skin-fold window chamber technique to assist in developing an *in vivo* model for HPV acquired tumor. Successfully establishment of mouse acquired tumor growth was shown in figure 13, 14. The histopathologic examination also confirmed that the human cervical cancer cells could grow in mouse tissue, in particular with the characteristic of squamous cell carcinoma (Figure. 17).

The different amount of HeLa cells were used and the results indicated that: 1) the size of tumor increased significantly in relation to the amount of transplanted cells, and 2) the rate of tumor growth (size/time duration) linearly increased in all three doses of HeLa cells (Figure 16). The results of intravital fluorescent videomicroscopy (Fig. 18) also indicated that at the site of implantation, the important process of tumor angiogenesis was existed within 2 weeks similar to other types of tumor implantation models [160]. The longest duration for these transplanted mice to stay alive was about 6 months after the inoculation.

Moreover, the developed tumor contained HPV-18 DNA was confirmed by using HPV DNA testing and genotyping. Therefore, the results strongly demonstrated that the existed tumor mass was definitely caused by HeLa cells growth. Although, several studies reported the development of animal models with HPV infection [161, 162], the methods used still have a lot of limitation and not practically represent for cervical cancer. In short, (1) the difficulty in the preparation of purified HPV viruses from infected tissue, (2) the need of human tissue as the starting materials used for viral inoculation before transplanting it into an animal, (3) most studies used the cutaneous HPV types, i.e., HPV-6 or 11, to induce tumor which is not the good representative of high risk types, i.e., HPV-16 or 18 causing cervical cancer, and (4) the beginning steps of tumor establishment such as increase vascularization or angiogenesis could not be observed in details. The advantages of the dorsal skin-fold window chamber technique in developing mouse bearing HPV tumor especially human cervical cancer cells are not only used for exploring the establishment steps of tumor but also for study the vaccine and drug efficacy specific to tumor. However, the difference of environmental factors

between cutaneous tissue and genital area should be considered and aware of. Moreover, we noticed that the transplanted mice using this technique always developed the necrotizing area and appearance of hematoma lesion similar to the previously reports when used xenograft techniques [163]

In conclusion, our novel *in vivo* model for HPV acquired cervical cancer is simply and highly reproducible in term of tumor induction model. This kind of animal model may be useful for screening and selecting candidate of novel agents and for the development of HPV infected-cervical cancer therapeutic treatment in the future experiments.

Although current standard therapeutic strategies for cervical cancer including surgery, radiotherapy or chemotherapy are usually effective for achieving initial disease control, the recurrent or metastasis patients still present. These patients usually are tolerant with standard treatment and associate with poor prognosis. This is a driving factor for many researchers to develop new strategies treatment for them. One strategy is mainly focused in to the field of immunotherapy. There are many attempts to develop DC based vaccination for cervical cancer. By this principle, autologous DC is pulsed with HPV antigens and then returned back into the patient, expecting that the DC will present an antigen to the effector lymphocytes (CD4+ T cells, CD8+ T cells). The activations are finally led to generate CTL response to specific HPV antigens on cervical cancer cells.

One observation of Nonn, M., *et al*, demonstrated that HPV-16 E7 and HPV-18 E7 proteins loaded on DC could induce specific CTL response to HPV antigens *in vitro* [115]. Chandy, A.G., *et al*, confirmed that DC based vaccination was able to eradicate tumor in mice bearing a HPV 16 antigen expressing tumor model [114]. Both examples rely primarily upon CMIR especially CTL response. However, CTL response specific to HPV antigens in cervical cancer patients is rarely demonstrated [164].

HPV is a genius DNA virus that has many pathways to escape the host immune response. HPV life cycle is strictly associated with proliferation and differentiation of epithelium cells. Viral proteins on epithelium cells surface are rarely or none expression. In addition HPV is able to down regulate of MHC expression on cell surface resulting in low antigen processing and presentation [88].

These problems emphasize the importance of developing immunotherapy strategies that do not require or even utilize classical MHC-restricted antigen processing and presentation. Several research groups back to the innate immune cells. One of them is the γ/δ T cells, the innate immune cells that recognize target cells in a MHC independent, requiring no processing or presentation of tumor specific antigens. Instead γ/δ T cells recognize a group of pyrophosphate antigens, non-peptide antigens which are expressed on stressed cells as well as tumor cells [137].

Several studies showed that γ/δ T cells especially Vγ9/Vδ2 T cells have ability to kill several tumor cell types both autologous and allologous cells such as pancreatic, hepatocarcinoma, adenocarcinoma, squamous cell carcinoma and lung carcinoma [28, 145, 165, 166]. However, no experiment in cervical cancer has been done. A few observation has been documented that increasing number of wart infiltrating γ/δ T cells can be observed in HPV-6 infected genital wart and suggested that this cell may have an important role in controlling of HPV infection [39].

Therefore, the role of Vγ9/Vδ2 T cells to cervical cancer cell lines was exploited. The γ/δ T cells were purified from PBMC of healthy blood donors. Before purification, PBMCs were stimulated with pamidronate and IL-2 (100 U/ml) to expand the γ/δ T cells population specific to pamidronate, a drug of choice in this study. The optimal concentration of pamidronate that could induce the highest expansion of γ/δ T cells in PBMC was 10 uM/ml (Figure 21) which approximately 77% of γ/δ T cells could be generated. The efficiency of γ/δ T cells purification was greater than 90 % (Figure20).

The evidence for the expansion of γ/δ cells by nBP drug was first revealed when increase numbers of γ/δ T cells were observed in patients who had acute-phase reactions after their first pamidronate treatment. [119]. This finding, led many researcher groups to use another derivatives of nBP drugs or synthetic pyrophosphate antigens for stimulating *ex vivo* expansion the γ/δ T cells. Among γ/δ T cells, Vγ9/Vδ2 T cells was the predominant subpopulation which usually ranging between 80-90% [28, 123]. In this study, approximately 87% of all expanded γ/δ T cells were Vγ9/Vδ2 T

cells (Figure 23). Stimulation PBMC with other synthetic pyrophosphate antigen to Vγ9/Vδ2 T cells for example bromohydrin pyrophosphate (BrHPP) or knows as phosphotim, also gave the same results. That is after 14 days of single stimulation, the percentage of Vγ9/Vδ2 T cells found ranging between 73.5-96.4% [139, 166].

Expansion of Vγ9/Vδ2 T cells could be stimulated by either pyrophosphate antigens or nBP drugs. Recognition of pyrophosphate antigens does not require antigen entry and processing. It could be directly presented to Vγ9/Vδ2 T cells [142]. In contrast to pyrophosphate antigens, nBP drugs use an indirectly mechanism to induce expansion of Vγ9/Vδ2 T cells. Inhibition of farnesyl pyrophosphate synthase (FPPS), down stream enzyme for converting IPP to other intermediate products, is one important mechanism for the functioning of bisphosphonate drugs. Therefore accumulations of IPP within treated PBMC population can be stimulate the expansion of Vγ9/Vδ2 T cells [35, 136]. This finding gives strong evidence in support that Vγ9/Vδ2 T cells could be expanded by induction of pamidronate or synthetic pyrophosphate antigen.

Vγ9/Vδ2 T cells directly recognize the IPP that express on the target cells and accumulations of IPP could be induced by pamidronate, therefore the accumulations of IPP within cancer cell lines could be induced by pamidronate. The optimal concentration of pamidronate that could induce accumulation of IPP within cervical cancer cell lines and not toxic to cells was 10 uM/ml (Table 4 and Figure 24), Here, the levels of accumulated IPP in cancer cell lines were not determined because of sophisticating detection. However, Gober, H.J., *et al* used HPLC technique to analyzed the accumulations of IPP in several zoledronate treated tumor cells [35]. .

In addition accumulation of IPP in tumor cells is a powerful danger signal that stimulates the Vγ9/Vδ2 T cells. Since only Vγ9/Vδ2 T cells can response to IPP antigen [167]. Thus, pamidronate treated cervical cancer cells must be sensitive to Vγ9/Vδ2 T cells recognition more than non-treated cells. Cytotoxic activity using LDH assay was performed. The results confirmed that pamidronate treated cervical cance cells (HeLa cell, SiHa cell, and CaSki cell) were sensitive to Vγ9/Vδ2 T cells (Table 5 and Figure 25). These indicated the possibility of IPP accumulation in the activated cells.

The most sensitive cell was HeLa cell followed by SiHa, CaSki, NTY and the less was PHA stimulated autologous PBMC. Although, the magnitude of killing might be different in each cell types, the trend of the killing in each individuals was the same (Table 5and Figure 25). This finding was similar to the study with other types of cancer. Corvaisier, M., *et al* indicated that purified Vγ9/Vδ2 T cells from colon cancer patient could kill autologous and allologous colon cancer cells with a different degree [33]. Many reasons to explain why different cancer cells were killed by Vγ9/Vδ2 T cells with a different degree were as followed.

Firstly, killing of target cells by Vγ9/Vδ2 T cells not only depend on the IPP antigen but also include with several surface molecules on the target cells and Vγ9/Vδ2 T cells. In the case of colon cancer, it also showed that recognition by Vγ9/Vδ2 T cells was highly dependent on IPP production and ICAM-I expression by target cells [33]. Another study, by screening the cell surface molecules expressed on several cancer cell lines was found that the expression of CD166, the adhesion

molecule of many cell types, closely paralleled with the capacity of Vγ9/Vδ2 T cells [168]. In addition, the cytotoxicity of γ/δ T cells, especially Vγ9/Vδ2 T cells is modulated by the multiple NK receptors expressed by γ/δ T cells such as NKG2D which expresses in different levels in γ/δ T cells subpopulations (70% in Vγ9/Vδ2 T cells, 18% Vγ9/Vδ1 T cells) [136]. The NKG2D is the specific receptor for human MHC class I-related molecules (MICA), which can be found on cell surface of many tumor cell types. However one study indicated down regulation of MICA could be observed in cervical cancer tissue [169] whereas another study demonated over expression of MICA on HeLa cells [170]. Therefore the difference levels of MICA expressed on each cervical cancer cell line may be one factor of killing activity of Vγ9/Vδ2 T cells.

Secondly, a different type of HPV infection in cervical cancer cell lines. The HeLa cell has HPV 18 DNA whereas; SiHa and CaSki have a different copies number of HPV-16 (SiHa 1-2 copies, CaSki 600 copies). The different of HPV subtype and copies number probably involve. Moreover, the abnormality of the mevalonate pathway with in cervical cancer cell lines may also play role. This is still unknown, however there are some evidences demonstrating that in lymphoma and leukemia cells have highly active HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway. Both of cancer cells were sensitive to Vγ9/Vδ2 T cells due to high level of IPP[136]. This is interesting issues to figure out the factors within the cancer cells in term of biochemical pathway factors or the role viral factors that may involve in responding of Vγ9/Vδ2 T cells.

The functions of Vγ9/Vδ2 T cells could be evaluated by cytokines releasing. Poccia, F.,*et al* demonstrated that several cytokines (such as GM-CSF, MIP-1 α/β, RANTES, TNF-α and IFN-γ) could be secreted from Vγ9/Vδ2 T cells after activating with IPP [171]. Among those cytokines, IFN-γ and TNF-α were the most important cytokines in responding to cancer. γ/δ T cells can play a necessary role in tumor immunity through provision of an early source of IFN-γ and TNF-α that in turn may regulate the function of tumor responded of α/β T cells [28, 32]. Thus, the levels of IFN-γ and TNF-α were the selected cytokines to described the functions of Vγ9/Vδ2 T cells in several studies [32, 33, 35, 145, 167]. Our results showed that IFN-γ and TNF-α releasing form stimulated Vγ9/Vδ2 T cells were correlated with cytotoxicity activity. The highest concentrations of IFN-γ and TNF-α were found in pamidronate treated HeLa cell, followed by SiHa, CaSki, NTY cell, whereas a little of IFN-γ could be measured from PHA activated autologous PBMC (Table 6-7.and Figure 26-27).

In our experiments IFN-γ could be detected at lease 5 h after co-culturing with the target cells corresponding to other studies indicated that IFN-γ was produced and secreted by Vγ9/Vδ2 T cells within 4-6 h [33, 145, 166]. Time release of cytokines of Vγ9/Vδ2 T cells (5 h) was short when it compared with the time release of CTL activity (12-48 h) [172, 173]. This indicated the rapid response of Vγ9/Vδ2 T cells as innate immunity versus acquired immunity. However, the releasing levels of both cytokines were quite variable, between individuals, and between types of cancer cells, implying that Vγ9/Vδ2 T cells from different individuals could response with a different degree to different cancer cells.

The important killing mechanism of γ/δ T cells to cancer cells is the induction of apoptosis which could be mediated by perforin and/or Fas/FasL pathway. Some studies demonstrated that the perforrin/granzyme was the main pathway that mediated apoptosis in renal cell carcinoma and human squamous cell carcinoma [139, 174]. To address the phenotypic functions of Vγ9/Vδ2 T cells we evaluated the expression of CD107, a marker associated with the degranulation pathway which involved it the perforin and granzyme mediated cell lysis. The results showed Vγ9/Vδ2 T cells were able to induce apoptosis via this mechanism (Figure 28). The data provides the information confirmed that cervical cancer cell lines could be killed by Vγ9/Vδ2 T cells. The killing activity of Vγ9/Vδ2 T cells mostly found in pamidronate treated cervical cancer cell lines while a little respond could be observed in non-treated cervical cancer cell lines.

Approximately 68% of Vγ9/Vδ2 T cells responsing to human squamous cell carcinoma could be previously demonstrated [174] but only 11% of all Vγ9/Vδ2 T cells responding to pamidronate treated HeLa cell, 14% in pamidronate treated SiHa cell, and 7% in pamidronate treated CaSki cell were detected in this study (Figure28). This implied that the specific clone of Vγ9/Vδ2 T cells may be involved in responding to cancer cell lines. According to the study of Corvaiser, M., *et al*, only specific clone of Vγ9/Vδ2 T cells, isolated from colon cancer ascites, was able to kill various autologous and allologous colon cancer cells. Therefore, it would be interesting for further study by isolating and characterization of the strong reactivity clone of Vγ9/Vδ2 T cells to cervical cancer cells.

Therefore, the role of Vγ9/Vδ2 T cells to cervical cancer cells in *vivo* was explored by using the previously established tumor mouse model [175]. Our results demonstrated that Vγ9/Vδ2 T cells deposited within the tumor sites after injection (Figure30) and could induce apoptosis within the cervical cancer cells *in vivo* (Figure 31). Our finding gives a strong support data to the tumor immunosurveillance role of Vγ9/Vδ2 T cells. Previously, a study in prostate cancer indicated adoptive transfer of syngenic mouse γ/δ T cells could localize the size of the tumor in mice and modulate the growth of established tumor [176]. The role of γ/δ T cells to cancer cells *in vivo* also showed in the study of Zheng, B.J., demonstrating that peripheral γ/δ T cells derived from healthy donors were able to exhibit cytotoxicity against nasopharyngeal carcinoma in mouse model [177].

Implantation of HeLa cells in to window chamber on the dorsal skin of BALB/c-nude mice can generates tumor in mice. Tumorigenesis confirmation by measuring the tumor size, anigiogenesis, hitopathology changes, and molecular detection of HPV DNA indicated tumor growth are originated from implanted HeLa cells. Approximately 90% of γ/δ T cells can be purified from healthy PBMC blood donors by using the MACS technique after stimulating with the optimized dose 10 uM/ml of pamidronate and 87% of Vγ9/Vδ2 T cells can be generated. *Ex vivo* expanded Vγ9/Vδ2 T cells are able to recognize and kill certain human cervical cancer cell lines *in vitro*. According to the hypothesized, pamidronate treated cervical cancer cells are sensitive to Vγ9/Vδ2 T cells than the non-treated cells. The most sensitive cell is HeLa cell followed by SiHa, CaSki, NTY and the less effective is PHA blast autologous PBMC. The cytotoxicity involves in releasing of IFN-γ and TNF-α was

demonstrated. The killing mechanism involves primarily the perforin/granzyme mediated pathway of granulation. Finally, *in vivo* study demonstrated that this Vγ9/Vδ2 T cells can induce apoptosis in tumor mouse model.

Our observations are strongly support the role of Vγ9/Vδ2 T cells in killing cancer cells. This is the first evidence indicated that Vγ9/Vδ2 T cells can be recognized and kills the cervical cancer cell line both *in vitro* and *in vivo*. Therefore, the Vγ9/Vδ2 T cells may be suitable candidates for the active or passive immunotherapy in recurrent or metastasis cervical cancer patients in further.

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APPENDICES

APPENDIX A

REAGENTS, MATHERIALS AND INSTRUMENTS

A. Media and Reagents

Agarose (ultrapure)	(ResearchOrganic,USA)
Anti-human FITC γ9 TCR	(BD Bioscience, USA)
Anti-human PE δ2 TCR	(BD Bioscience, USA)
Anti-hapten FITC Microbeads	(Miltenyi, Germany)
Anti-γ/δ TCR	(Miltenyi, Germany)
Anti-Human FITC γ/δ TCR	(BD Bioscience, USA)
Anti CD107-FITC	(BD Bioscience; USA)
CellTracker CMFDA	(Invitrogen; USA)
DNA extraction kit	(Qiagen, USA)
EDTA	(Biobasic, Canada)
Fetal Bovine Serum	(PAA, Austria)
FITC-dextran	(Sigma, USA)
IFN-γ ELISA detection kit	(Peprotech, USA)
In situ apoptosis detection kit	(Chemicon, USA)
INNO-LiPA HPV detection kit	(INNo-LiPA,Germany)
IL-2	(Peprotech, USA)
LDH cytotoxicity	(Roche, USA
Lymphoprep	(Axis-Shield,Norway)
MEM medium	(Gibco BRL, USA)
Magnetic Activating cell sorter	(Miltenyi, Germany)
Pamidronate	(Novartis, USA)
Patassium phosphate	(Biobasic, Canada)
Pentobarbital sodium	(CEVA, France)
Penicillin	(Biobasic, Canada)
Potassium chloride	(Merck, Germany)

Rabbit anti-Human CD3	(DAKO, USA).
RPMI medium 1640	(PAA,Austria)
Sodium phosphate	(Biobasic, Canada)
Streptomycin	(Biobasic, Canada)
Taq DNA polymerase	(Fermentus, U.S.A.)
TNF-α ELISA detection kit	(Peprotech, USA)
Tris Base	(ResearchOrganic,USA)
Trypan blue	(Sigma, UK)
Trypsin	(Biobasic, Canada)

B. Materials

Centrifuge tube	(Labcon, Germany.)
Disposable serological pipette	(Labcon, Germany.)
ELISA plate	(Millipore, U.S.A.)
Filter Tip	(Labcon, Germany.)
Microcentrifuge tube	(Sorenson, U.S.A.)
24-well flat plate	(Costar, USA)
96-well polyvinylidene difluoride backed plates	(Millipore, Bedford, MA)
Tissue culture flask	(Nunc,Denmark)

C. Instruments

Autoclave (model-SX-700)	(Tomy, Japan)
CO_2 Incubator	(Thermo Forma, U.S.A.)
DNA thermocycle system	(Hybaid, U.S.A.)
Electrophoresis chamber	(CBS, U.S.A.)
Microcentrifuge	(Fotodyne, U.S.A.)
Power supply (Model 1000/500)	(Bio-Rad, U.S.A.)
Refrigerator	(Toshiba, Japan)
Spectrophotometer (SmartSpect™ 3000)	(Bio-Rad, U.S.A.)
Water bath	(Julabo, Germany)
Micro plate reader	(Humanreader, Germany)
Flow cytometer	(BD Bioscience; USA).

APPENDIX B

REAGENTS PREPARATION

Reagents for cells cultivation, PBMC cultivation and isolation, Flow cytometry and tissue fixation

1. **Lymphoprep solution** (ready to use)

2. **RPMI medium 1640** (ready to use)

3. **Penicillin 100,000 Units/ml**

1,000,000 Unit Penicillin G	1	ampoule
Deionized Distilled water	10	ml

Sterilized by filtration and stored at -20℃

4. **Streptomycin 100,000 µg/ml**

1 gm Streptomycin	1	ampoule
Distilled water	10	ml

Sterilized by filtration and stored at -20℃

5. **1 M HEPES**

HEPES	23.83	g
Deionized distilled water to	100	ml

Sterilized by autoclaving and stored at 4℃

6. **10 % $NaHCO_3$**

$NaHCO_3$	10	g
Deionized distilled water to	100	ml

Sterilized by autoclaving and stored at 4℃

7. R10

RPMI 1640	900	ml
100,000 unit/ml Penicillin G	1	ml
100,000 µg/ml Streptomycin	1	ml
Fetal Bovine Serum (FBS)	100	ml
1M HEPES	1	ml
10 % $NaHCO_3$	2	ml

Stored at 4℃

8. Growth medium

MEM medium	90	ml
100,000 unit/ml Penicillin G	1	ml
100,000 µg/ml Streptomycin	1	ml
Fetal Bovine Serum (FBS)	100	ml
1M HEPES	1	ml
10 % $NaHCO_3$	2	ml

Stored at 4℃

9. 5% Trypsin

Trypsin	5	g
Deionized distilled water to	100	ml

Sterilized by filtration and stored at -20℃

10. 1**% EDTA**

EDTA	1	g
Deionized distilled water to	100	ml

Sterilized by filtration and stored at 4℃

11. Trypsin/EDTA (0.025% trypsin, 0.01% EDTA)

5% Trypsin	0.5	ml
1% EDTA	0.2	ml
1X PBS up to	100	ml

Do not autoclave

Sterilized by filtration and stored at 4℃

12. 10 X PBS

NaCl	80	g
KCL	2	g
$NaHPO_4$	11.5	g
KH_2HPO_4	2	g
Deionized distilled water to	900	ml

Adjusted to pH 7.4 and adjusted volume to
1000 ml with additional deionized distilled water
Sterilized by autoclaving and stored at room temperature

13. 1X PBS

10 X PBS	100	ml
Deionized distilled water	900	ml

14. Neutral buffer formalin

Formalin (40% formaldehyde)	100	ml
Sodium phosphate, monobasic, monohydrate ($NaH_2 HPO_4 H_2O$)	4	g
Sodium phosphate, dibasic, anhydrous ($Na_2 HPO_{4)}$	6.5	g
Deionized distilled water to	1000	ml

15. citrate PBS buffer

Sodium citrate	4.5	g
1X PBS to	1000	ml

Sterilized by autoclaving and stored at 4℃

16. Fixation buffer for Flow cytometry

2% Formaldehyde	12.5	ml
0.2 % sodium azide	0.5	ml
1X PBS	37	ml

Reagents for molecular analysis

1. **TE buffer (Tris/EDTA)**

Tris, PH 7.4	10	mM
EDTA, pH 8.0	1	mM

2. **10 mg/ml Ethidium bromide**

Ethidium bromide	1.0	g
Sterile distilled water	100	ml

Mix the solution and sore in the dark at 4°C.

3. **1.5% Agarose gel**

Agarose	0.325	g
0.5X TBE	35	ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

APPENDIX C

FIGO staging of cervical carcinomas

Stage I

Stage I is carcinoma strictly confined to the cervix; extension to the uterine corpus should be disregarded. The diagnosis of both Stages IA1 and IA2 should be based on microscopic examination of removed tissue, preferably a cone, which must include the entire lesion.

Stage IA: Invasive cancer identified only microscopically. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm.

Stage IA1: Measured invasion of the stroma no greater than 3 mm in depth and no wider than 7 mm diameter.

Stage IA2: Measured invasion of stroma greater than 3 mm but no greater than 5 mm in

depth and no wider than 7 mm in diameter.

Stage IB: Clinical lesions confined to the cervix or preclinical lesions greater than Stage

IA. All gross lesions even with superficial invasion are Stage IB cancers.

Stage IB1: Clinical lesions no greater than 4 cm in size.

Stage IB2: Clinical lesions greater than 4 cm in size.

Stage II

Stage II is carcinoma that extends beyond the cervix, but does not extend into the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.

Stage IIA: No obvious parametrial involvement. Involvement of up to the upper twothirds of the vagina.

Stage IIB: Obvious parametrial involvement, but not into the pelvic sidewall.

Stage III

Stage III is carcinoma that has extended into the pelvic sidewall. On rectal examination, there is no cancer-free space between the tumour and the pelvic sidewall. The tumour involves the lower third of the vagina. All cases with hydronephrosis or a non-functioning kidney are Stage III cancers.

Stage IIIA: No extension into the pelvic sidewall but involvement of the lower third of the vagina.

Stage IIIB: Extension into the pelvic sidewall or hydronephrosis or non-functioning kidney.

Stage IV

Stage IV is carcinoma that has extended beyond the true pelvis or has clinically Involved the mucosa of the bladder and/or rectum.

Stage IVA: Spread of the tumour into adjacent pelvic organs.

Stage IVB: Spread to distant organs.

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